



Characterisation of Drug-Specific T-Cell Responses in Hypersensitive Patients and Healthy Donors

This thesis is submitted in accordance with the requirements of the University
of Liverpool for the degree of Doctor of Philosophy by

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Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree



.....

Zaid Al-Attar

M.B.Ch.B, M.Sc. (Pharmacology)

To:

My parents

and

My family

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Abbreviations:

aa	Amino acids
AB serum	Human serum from type AB donors
ADCC	Antibody dependent cellular cytotoxicity
ADR	Adverse drug reaction
AICD	Activation induced cell death
ALP	Alkaline phosphatase
APC	Antigen presenting cell
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
BSA	Basophil activation test
BSA	Bovine serum albumin
BTLA	B- and T-lymphocyte attenuator
CBZ	Carbamazepine
CCR	CC chemokine receptors
CD	Cluster of differentiation
CDR	Complementarity determining region
CF	Cystic fibrosis
CFSE	Carboxyfluorescein succinimidyl ester
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CI	Confidence interval
CLA	Cutaneous lymphocyte-associated antigen
Cmax	Maximum (or peak) serum concentration that a drug achieves in a specified compartment or test area of the body after the drug has been administrated and prior to the administration of a second dose.
Cpm	Count per minute
CSA	Cyclosporin-A
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CXCR	CXC chemokine receptors
DC	Dendritic cell
DH20	Distilled water
DHR	Drug hypersensitivity reaction
DMSO	Dimethyl sulfoxide
DPT	Drug Provocation Test
DRESS	Drug Reaction (or Rash) with Eosinophilia and Systemic Symptoms
EAP	Extended antigen priming
EBV	Epstein Barr virus
EBVs	B-lymphoblastoid cell lines transformed by Epstein-Barr virus (EBV)
E-Cad	E-cadherin

EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ESI	Electron electrospray ionization
Fab	Fragment antigen-binding
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable region
FDE	Fixed drug eruption
FITC	Fluorescein isothiocyanate
FSC	Forward scattering
GSH	Glutathione
GSTP	Human GSH S-transferase
Gy	Gray (unit) SI unit of absorbed radiation
HBSS	Hank's balanced salt solution
HPLC	High-performance liquid chromatography
HVEM	Herpesvirus entry mediator
I.V.	Intravenous
ICOS	Inducible T-cell costimulator
ICS	Intracellular Cytokine Staining
IDILI	Idiosyncratic Drug-Induced Liver Injury
IDR	Idiosyncratic drug reaction
ITAM	Immunoreceptor tyrosine-based activation motifs
LAT	Linker for T-cells activation
LCK	Lymphocyte-specific protein tyrosine kinase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
mAb	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MFI	Mean fluorescence index
Motif	A common sequence in a peptide that binds to the MHC molecule
MPE	Maculopapular exanthema
MRM-MS	Multiple Reaction Monitoring MS
MS	Mass spectroscopy
NIRs	Non immediate allergic reactions
NK	Natural killer cell
nL/min	Normal litres per minute
NSAIDS	Non-steroidal anti-inflammatory drugs
PAMP	Pathogen associated molecular pattern
PBMCs	Polymorph nuclear blood cells
PBS	Phosphate-buffered saline

PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PHT	Phynetoin
Pip	Piperacillin
PMN	Poly-morphonuclear leukocytes
PS	Penicillin and streptomycin
PVDF plate	Polyvinylidene Fluoride plate (for ELISPOT)
PZA	Pyrazinoic acid
SFU	Spot forming unit
SI	Stimulation index
SJS	Steven Johnson syndrome
SLP76	SH2-domain containing leukocyte protein of 76kDa
SMX	Sulfamethoxazole
SMX-NO.	Sulfamethoxazole-nitroso
SSC	Side scattering
STAT3	Signal transducer and activator of transcription 3
TAP	Transporter associated with the antigen presentation
TCC	T-cells clones
Tcm	Central memory T-cells
TCR	T cell receptor
Teff	Effector T-cells
Tel	Telaprevir
Tel-m	Telaprevir metabolite (VRT-127394)
Tem	Effector memory T-cells
TEN	Toxic epidermal necrolysis
TNF-α	Tumor necrosis factor alpha
ZAP-70	ζ-chain-associated protein kinase

Characterisation of Drug-Specific T-Cell Responses in Hypersensitive Patients and Healthy Donors

Zaid Al-Attar

Drug hypersensitivity reactions represent a significant clinical problem and an impediment to drug development. It is currently impossible develop drugs with no immunological liability; furthermore, it is very difficult to predict which individuals will develop hypersensitivity when exposed to a therapeutic treatment regimen. This is because susceptibility is a function of the chemistry of the drug, the genetic background of the patient and environmental factors such as patient demographics, disease and concomitant medications. In this study, we focused on two drugs: piperacillin and telaprevir as examples of idiosyncratic hypersensitivity reactions.

Regarding piperacillin, we characterized the piperacillin reactive T-cells response of piperacillin hypersensitive patients with cystic fibrosis in terms of proliferation, cytokine secretion in addition to TCR-V β and chemokine receptor expression. Piperacillin-responsive CD4⁺ clones expressing a diverse TCR-V β s proliferated and secreted Th1/Th2 cytokines in a dose-dependent manner. Clones expressed chemokine receptors consistent with a mixed Th1/Th2 response. Piperacillin responsive T-cells displaying a similar phenotype were also generated from naïve healthy volunteers using a dendritic cell-T-cell priming technique. However, TCR-V β expression was more restricted; high expression of TCR-V β 9, 13.2, 18 and 4 was detected. Piperacillin is β -lactam antibiotic that is known to covalently modify lysine residues on proteins such as human serum albumin. Therefore, we characterized the absolute levels of piperacillin protein binding in patients and in vitro culture and whether piperacillin albumin adducts activate patient T-cells. With the aid of mass spectrometry, we identified the main lysine residues that piperacillin modifies and synthesized a piperacillin modified peptide incorporating amino acid residues of albumin to establish a standard curve for quantification of binding. The level of modified Lys541 ranged from 2.7-4.7% in patients. Analysis of incubation medium from piperacillin-responsive clones revealed that a similar level of piperacillin-modified Lys541 in albumin was required for the stimulation of T-cells. Antigen presenting cells cultured with piperacillin for 24h also activated the clones with 2.8% Lys541 modification at this time-point. Piperacillin-albumin conjugates that had levels and epitopes identical to those detected in patients were synthesized and purified, and were shown to stimulate T cells in an antigen processing dependent manner. Piperacillin-albumin conjugate clones expressed a restricted pattern of TCR-V β with high expression of TCR-V β 9 in many clones.

Telaprevir is antiviral drug used for the management of hepatitis C. We succeeded in generating clones responsive to a telaprevir metabolite (VRT-127394) using PBMCs priming technique and these clones were found to be 100% cross reactive with telaprevir. These clones were found to be activated via the direct binding of the drug (metabolite) to MHC molecules on antigen presenting cells. Most clones were CD4 T-cells and they displayed a restricted pattern of TCR-V β expression.

Together, our results define in immunological, chemical and quantitative terms the drug immune receptor interactions that can drive a T-cell response. For β -lactam antibiotics, the levels of modification that activated T-cells *in vitro* are equivalent to the ones formed in patients.

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Chapter One

General Introduction

1 General Introduction:

In the last few decades, there was an increased burden from the problem of drug allergy especially in the developing countries. This issue has received a great deal of focus in the form of research effort and funding in order to discover and establish a comprehensive understanding of the mechanisms involved in immunological reactions. Collectively, the research was conducted with a deep recognition of the final target, which was the development of new therapeutic agents to combat the rising problem of drug allergy. Moreover, it is crucial to explore in depth to modify the current modalities of management and treatment to lessen the adverse drug reactions in general, and specifically drug hypersensitivity reactions.

The aim of this chapter is to present a review about the main topics in immunology, pharmacology and drug-induced reactions.

1.1 Adverse reaction and adverse event

Adverse event is unpleasant or harmful effect or symptoms that may be associated with the administration of a drug or at some time later but may not be necessarily a result of the mechanism of drug action (Pirmohamed et al., 1998). In fact, it occurs just as a coincidence with no degree of probability or attribution e.g. accident, unintended pregnancy.

On the other hand, the mechanism of drug action is the pivotal factor for **adverse drug reactions (ADR)**. In general, adverse reaction can always be regarded as an adverse event but the reverse is not true always. This division between those two terms which was first recognized by Finney (2006) is crucial especially in clinical trials where researchers may use the term “events” to denote consequences that are not attributed to the drug (Finney, 2006).

1.2 Adverse drug reaction

“An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, usually predicting hazard from future administration and warranting prevention, or specific treatment, or alteration of the dosage regimen, or withdrawal of the product” (Aronson, 2011). One metanalytic study showed that the frequency of ADRs in hospitalized patients is 16.88% (CI95%: 13.56,20.21%) (Miguel et al., 2012). Another study has shown that ADRs are responsible for 6.5% of admissions to hospitals with a median bed stay of 8 days (Pirmohamed et al., 1998;Williams et al., 2004).

There is a high burden on the health services in terms of financial expenses which were estimated to be 466 million pounds annually in the UK (Pirmohamed et al., 2004). ADRs are a major cause for the withdrawal of drugs and it takes an average of 10 yrs to develop a new drug with a cost reaching approximately 866 million pounds (Adams and Brantner, 2006).

Therefore, there is a need to understand mechanisms of ADRs so that they can be predicted during early stages of research and development.

1.3 Classification of ADRs

The most widely used classification is the A/B classification, which is centred mainly on the recognized relationship between drug dose and the development of an adverse effect.

1.3.1 Type A reactions:

(Sometimes called: **On target**) They constitute (80% of ADRs) (Shah et al., 2012). They are defined as adverse effects that result as an exaggerated response of the pharmacological effect of the drug. They are dose related, reversible by lowering the dose or stopping the drug, predictable and rarely lethal (Page, 2006;Park et al., 2010;McDowell et al., 2011).

Type A reactions are subdivided according to the classification of Rawlins and Thompson which is the most widely used classification system (Shah et al., 2012; Rawlins and Thompson, 1991):

1.3.1.1 Toxicity or overdose

Occurs as a result of an exaggeration of the drugs primary pharmacological action, which leads to cell damage at high doses. However, toxicity may occur at normal or low doses due to an underlying abnormality in the patient (e.g. hepatic failure with therapeutic doses of paracetamol) (Bennett et al., 2012).

1.3.1.2 Side effects

"Any effect caused by a drug other than the intended therapeutic effect, whether beneficial, neutral or harmful" (e.g. sedation with antihistamines) (Ferner and Butt, 2008).

1.3.1.3 Secondary effects

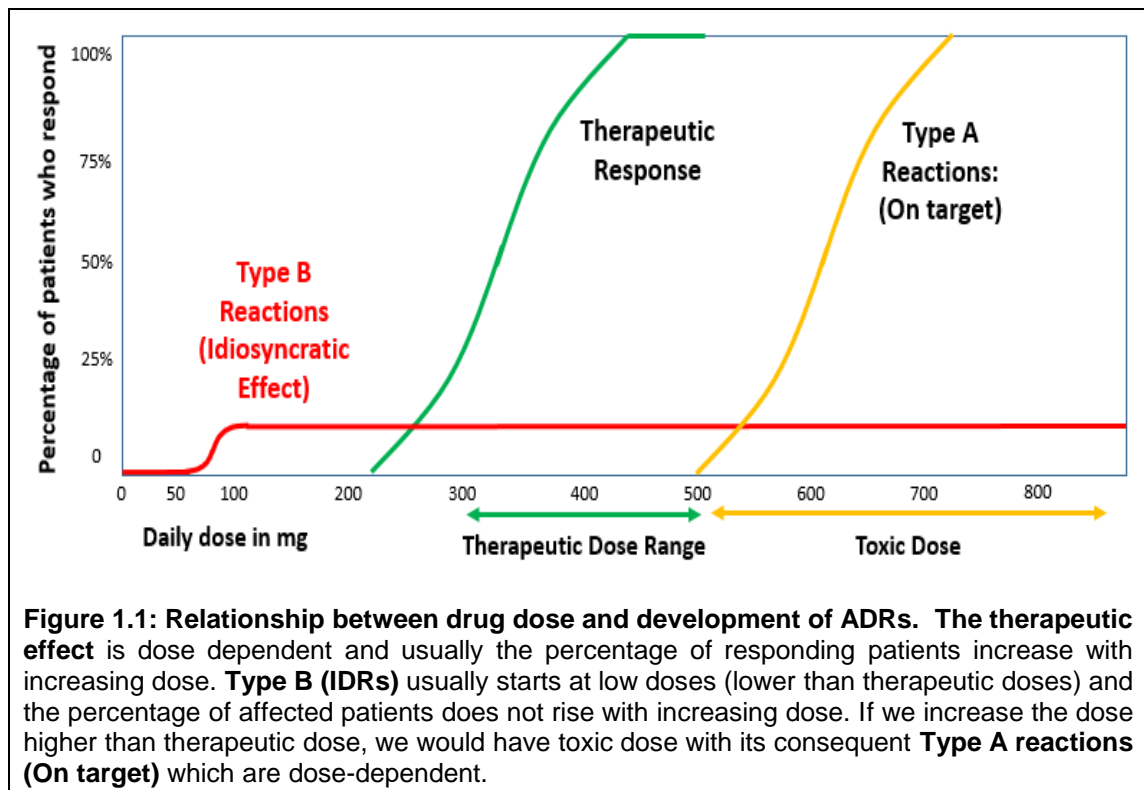
Are indirect net results of the primary action of the drug (e.g. development of diarrhea with antibiotic therapy due to altered gastrointestinal bacterial flora) (Shah et al., 2012).

1.3.1.4 Drug Interaction

Pharmacological reaction caused by the administration of a drug combination that is different from the predicted effect of each drug (e.g. theophylline toxicity in the presence of erythromycin therapy) (Shah et al., 2012; Helms and Quan, 2006).

1.3.2 Type B reactions:

(Sometimes called: **idiosyncratic, off-target**) They constitute (20% of ADRs) (Shah et al., 2012). Unusually are unpredictable, and cannot be explained by the



pharmacology of the drug. Instead, they are explained by pathogenesis which often relates to an inadvertent activation of the hosts immunological system by the drug. Moreover, their occurrence has no clear relationship with the dose (**Figure 1.1**). **Idiosyncratic drug reactions (IDRs)** are often described as dose independent. This is not a precise description. Actually, the probability that a drug may cause IDRs is related to the therapeutic dose of the drug. Drugs given at a dose less than 10mg/day rarely cause IDRs (Uetrecht, 2007) and 77% of 598 cases of liver IDRs were found to be caused by drugs given at doses higher than 50mg/day (Lammert et al., 2008). What is true is that most patients would not develop any IDRs at any dose, and there may not be a significant difference of the incidence of IDRs in the narrow range of doses used therapeutically. Moreover, the required dose to trigger an IDRs may be lower in a patient who has been previously exposed to the drug. Nevertheless there will be always a dose that below which no one would develop an IDRs (Zhang et al., 2011).

These reactions are usually delayed in onset and this is variable with the type of the IDR and even with the type of drug. However, IDRs tend to occur almost immediately on re-challenge. Surprisingly, the same drug can produce different types of IDRs.

Type B reactions constitute a great burden on the pharmaceutical industry since they add a significant uncertainty to drug development, which is translated in the form of extra time and additional cost. Furthermore, there is a great risk of candidate failure (B.K. Park et al., 2010).

Type B reactions are subdivided according to classification proposed by Rawlins and Thompson (1991) into:

1.3.2.1 Non-Immunological:

Which are divided into:

A. Intolerance: Lower threshold for the pharmacodynamic effect of the drug in comparison to the normal population (e.g. tinnitus with use of Aspirin) (Bennett et al., 2012).

B. Pseudoallergic (Nonallergic Hypersensitivity):

These reactions are characterized by a direct drug interaction with inflammatory cells; in particular, mast cells, eosinophils and basophils. There is no evidence of a role for the adaptive immune response (Pichler et al., 2015).

In most of the cases such reactions are genetically determined and mediated by mechanisms that entail the endogenous release of biologically active substances like histamine and leukotrienes (Bennett et al., 2012). Other mechanisms may be implicated like complement activation, which may trigger mast cell activation (Pichler, 2007). In some of these reactions the drug interacts with MRGPRX2 receptor on mast cells leading to IgE-independent mast cell degranulation (McNeil et al., 2014).

Remarkably, some patients may develop increased tryptase levels (proteinase enzyme contained in mast cells and plays an important role in allergic reactions); however, subsequent diagnostic tests do not show mastocytosis (Pichler et al., 2010). Moreover, traditional provocation tests often yield negative results (Aronson, 2011).

A prominent feature of pseudoallergic reactions that are caused by non-steroidal anti-inflammatory drugs (NSAIDS) is that they require higher doses and they arise slower in comparison to true IgE mediated reactions (more than 15 minutes) (Pichler, 2007). Pseudoallergic reactions that simulate type I reactions are called “anaphylactoid” e.g. morphine , tubocurarine (Bennett et al., 2012).

*(Note: Types of hypersensitivity reactions (I-IV) are discussed under the **heading 1.3.2.2**).* Regarding therapeutic approach, some mild pseudoallergic reactions have shown good response to antihistamines, but it is questionable whether reactions that are more serious will respond to antihistamines or corticosteroids (Bennett and Gracey, 2000;Pichler et al., 2010).

In addition to anaphylactoid reactions, there are additional pseudoallergic reactions, which mimic other forms of immunological reactions:

Type II reactions may be mimicked in patients with drug-induced hemolysis e.g. sulfonamides.

Type III reactions may be mimicked in patients with penicillamine-induced nephropathy (Bennett et al., 2012).

C. Metabolic idiosyncratic reactions: These reactions are an inherent abnormal reaction to a drug that does not occur in most patients at usually used clinical doses. Furthermore, these reactions do not involve the known pharmacology of the drug. They are usually mediated by reactive drug metabolites that are mostly generated by the liver (Uetrecht, 2008). In some cases there is evidence that susceptible patients are predisposed by a genetic abnormality (Bennett et al., 2012). In liver, the enzymatic system of cytochrome P450 plays an important role

in metabolizing drugs and generating reactive metabolites. An important example is isoniazid, in which polymorphisms in metabolic enzymes (in this case N-acetyltransferase and cytochrome P450 2E1) constitutes to be a risk factor for idiosyncratic liver toxicity (Huang et al., 2002). As a term, metabolic idiosyncrasy often used to describe specific forms of drug-induced hepatitis and cytopenias such as agranulocytosis (Guest et al., 1998). Several criteria are used to differentiate these reactions from the immune-mediated reactions. For example, if hepatotoxicity is associated with fever, rash, eosinophilia, antidrug antibodies with rapid occurrence after re-challenge, it is generally presumed that the reaction is immunological. In contrast, if these criteria are not present then most likely, the reaction is metabolic idiosyncrasy. This method of delineation is widely accepted by hepatologists (Uetrecht, 2008).

1.3.2.2 Immunological (Drug Allergy)

Two researchers, Coombs and Gell, working in the UK in the 1963 assembled the key facts about allergic reactions into four clearly defined pathological processes and essentially established the first classification system for drug allergy (Gell and Coombs, 1963):

Type I: IgE antibody mediated hypersensitivity, due to binding of the allergen to IgE on the surface of mast cells and basophils which causes the release of chemical mediators such as, histamine, kinins, prostaglandins and slow reacting substance of anaphylaxis with the net result of increased vascular permeability, vasodilation, smooth muscle contraction and oedema (Grant et al., 2007). These changes translate to the clinical presentation of urticaria, anaphylactic shock and asthma. This type of hypersensitivity develops within minutes and may last for 1-2 hr and sometimes reactions are fatal (Bennett et al., 2012).

Type II: antibody-antigen reaction involving IgG or IgM, which causes cell destruction by complement activation or by the cytotoxic effect of natural killer cells bearing Fc-IgG receptors. Other cells may mediate a similar effect. These include: macrophages, eosinophils and neutrophils. Cell lysis is mediated via

lysosomal enzymes and perforin e.g. Good-Pasteur syndrome (Pichler et al., 2010).

Type III: IgG mediated reaction. The end result is antibody-antigen immune complex deposition with subsequent tissue damage mediated by chemotaxis of polymorphonuclear leukocytes and natural killer cells with lysosomal enzyme release e.g. Serum sickness, Arthus reaction, heparin induced thrombocytopenia (Cruse et al., 2004).

Type IV: Cell mediated immunity, which is mediated by T-helper and T-cytotoxic cells (Aronson, 2011). Type IV reactions have been subdivided according to the cytokines and type of cells involved into four subdivisions (**Table 1.1**):

Table 1.1: Subdivision of type IV hypersensitivity reactions. Adapted from (Posadas and Pichler, 2007).				
	T-cell phenotype	Immune reactant	Effector cells	Clinical symptoms (example)
Type IVa	Th1	IFN-γ, TNF-α	Monocyte / macrophage activation	contact dermatitis, bullous exanthema
Type IVb	Th2	IL-5, IL-4, IL-13, eotaxin	T-cells driving eosinophilic inflammation	Maculopapular and bullous exanthema,
Type IVc	Cytotoxic T-cells	perforin, granzyme B, Fas ligand	CD4 + /CD8 + mediated T-cell killing	Contact dermatitis maculopapular, bullous exanthema,
Type IVd	T-cells	GM-CSF, CXCL-8	T-cell leading to recruitment and activation of neutrophils	Pustular exanthema

The peculiar feature of the **A/B** classification is the opportunity to use the alphabetical abbreviations to denote more expressive terms i.e. **augmented** and **bizarre** (Page, 2006). This classification was expanded later by the addition of another four divisions to become a six-category classification (A-F).

These new divisions are denoted:

C: Chronic, for ADR that manifests after prolonged exposure and not with single dose. **D: Delayed**, for ADRs that are remote from the onset of therapy e.g. in the treated patients many years after treatment or in their offspring.

E: End of use, for ADR that occurs after stopping the drug (especially if stopped suddenly, so-called withdrawal reaction).

F: Failure, failure of treatment, which is usually caused by drug interactions (Scott and Thompson, 2011).

In addition to the previously mentioned classification system which relies mainly upon immunological mechanisms there is also a clinical classification by Levine (1966); by which the ADRs are classified according to time factor (the time between drug administration and the start of the immunological reaction) into three groups: **immediate** (<1hr), **accelerated** (1-24hr) and **delayed** (>24hr).

More recently, to make it more convenient and practical, the accelerated and the delayed types were combined together into a single form, which is called: **non-immediate reactions (NIRs)**.

Immediate reactions (<1hr) are mediated by IgE antibodies and occur usually within minutes of drug administration. They present in the form of angioedema, urticaria and/or anaphylaxis (Romano et al., 2004). In contrast, **non-immediate reactions** (>1hr) are T-cell mediated. They may be manifested within hours, days or even weeks and are versatile in their clinical presentation (Park et al., 2010).

1.4 Drug Allergy

The first use of the term “allergy” was made by the Austrian scientist Clemens von Pirquet who had noted in 1906 that patients who were receiving the 2nd dose of horse serum were developing quicker and more severe reactions compared to the 1st dose (Silverstein, 2000).

Allergy as a term and its related clinical manifestations e.g. anaphylaxis and hypersensitivity have greatly evolved over the decades since the initial observations of Clemens von Pirquet.

The last updated definition of allergy was presented by EAACI: (European Academy of Allergology and Clinical Immunology) in 2001 and was advocated by World Allergy Organization (WAO) in 2003. The update in the terminology and definition is founded upon the mechanism of the reaction. According to this classification allergy is defined as “a hypersensitivity reaction initiated by specific immunologic mechanisms” (Johansson et al., 2003). Allergy could be IgE mediated or non-IgE mediated. In some conditions where other mechanisms have been proven e.g. hypersensitivity to aspirin, the term “pseudoallergic (nonallergic) hypersensitivity” is applied (Stevenson and Szczeklik, 2006; Li et al., 1991).

The most recent definition for a hypersensitivity reaction is “objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons” (S.G.O Johansson et al., 2003). In comparison with the previous terms, the term “anaphylaxis” is more clinical and commonly used by physicians. It states, “Anaphylaxis is a severe, life-threatening generalized or systemic hypersensitivity reaction”. When immunological mechanism such as IgE immune complexes are the culprit in anaphylactic reaction, the term “allergic anaphylaxis” is used. Accordingly, peanut induced food anaphylaxis may be denoted as “IgE-mediated allergic anaphylaxis”, whereas anaphylaxis provoked by non-immunological mechanisms is usually classified under the term “pseudoallergic (nonallergic) anaphylaxis” (Johansson et al., 2003; Aronson, 2011).

1.4.1 Mechanisms of T-cell-mediated Drug Allergy

Over the last 20 years, several hypotheses have been postulated to explain the development of drug allergy. However, the main ones that have wide acceptance are **hapten/prohapten** and **pharmacological interaction (p-i) mechanisms**. In

addition to these major mechanisms, there is a growing recognition that other mechanisms may be involved. For example, an immune-mediated reaction can be initiated by direct activation of antigen presenting cells, by alteration in the immune tolerance, and by epigenetic effects.

However, it should be noted that these mechanistic classifications are designed to simplify the complex processes that occur in vivo. In other words, the immune system combines several mechanisms to deal with a real or putative antigen. Nevertheless, the clinical picture usually reflects the dominance of a particular mechanism over the others (Pichler, 2007).

1.4.1.1 The Hapten/pro-hapten hypothesis

The traditionalist view of drug hypersensitivity dictates that drug-modified proteins are taken up by antigen presenting cells and processed into peptide fragments prior to presentation in the context of major histocompatibility complex (MHC) to T-cells. The first notion for this hypothesis came from Landsteiner who in 1935 reported that it was not possible to trigger an immune response to small molecules unless such molecules are chemically reactive and bound to a protein (Landsteiner and Jacobs, 1935). The hapten hypothesis assumes that small molecules (less than 1000 Da) are unable to elicit immune response due to poor binding between such drug molecules and MHC proteins. Furthermore, if the binding between the drug molecule and the protein is reversible, such conjugates would be not successful in inducing an immune reaction (Uetrecht, 2008).

According to hapten hypothesis, a **chemically reactive** drug (or active metabolite) binds **covalently** to a **protein** (which could be extracellular like albumin or cell membrane protein like integrin or intracellular protein like enzymes), and then this new product triggers an immune response against the altered self-protein (Pichler, 2007).

T-cells sensitization occurs when drug-protein adducts are taken by APCs. In the presence of an appropriate cytokine micro-environment, the APCs travel to the

draining lymph nodes where these adducts are processed and presented by MHC molecules to the T-cells receptors (TCRs) on naïve T-cells. Then, naïve T-cells with appropriate specificity will be stimulated undergo clonal expansion (Naisbitt et al., 2000). Primed T-cells can evolve into functionally distinct subclasses: Effector T-cells (T_{eff}), effector memory T-cells (T_{em}) which migrate to the peripheral tissues where the drug-protein adduct originated (Schnyder and Pichler, 2009). In contrast, central memory T-cells (T_{cm}) migrate to the draining lymph nodes (Ebert et al., 2005).

Drug adducts that are processed and presented as hapten-peptides to T_{eff} and T_{em} would result in local T-cell mediated inflammation, while drug-peptide presentation to T_{cm} in the draining lymph nodes would manifest in enlargement of the regional lymph nodes (Schnyder and Pichler, 2009).

Following the work of Landsteiner proteins were shown to be processed and presented as small as 8-20 amino acids (aa) long peptides by the MHC molecules. Twelve human leucocyte antigen (HLA) alleles are encoded per individual. The respective MHC proteins present different peptides (mostly 8-10 aa for HLA class I, 14-20 aa for HLA class II) which fit into the peptide binding groove of the HLA molecule (Weltzien et al., 1996). Later work by Weltzien et al have shown that the location of the hapten modification has a influences the development of an antigen-specific T-cell response (Weltzien et al., 1996).

The hapten hypothesis coincides with the classical “self” and “nonself” theory in that the immune system identifies drug-modified proteins as foreign antigens (Koen et al., 2006).

It is important to mention that the first drug reaction studied and proven to support this hypothesis was penicillin allergy. The presence of an inherent reactive β -lactam ring means that penicillin reacts irreversibly with free lysine amino acids on proteins. Consequently, this results in the generation of penicillin modified proteins that stimulate IgE antibody production and the activation of T-cells. Penicillin-specific IgE antibodies have been shown to provoke allergic reactions

through stimulating the degranulation of mast cells and basophils to release chemical mediators such as histamine, leukotrienes etc.

The other possibility is that lysine modified peptides would be recognized as neo-epitopes for activating reactive T-cells (Brander et al., 1995).

Although, the hapten hypothesis has established a framework for studying allergic drug reactions, it should be kept in mind that the presence of antidrug antibodies does not per se indicates that an ADR is mediated by these antibodies or that this ADR is immune mediated (Uetrecht, 2008).

Not every hapten modification leads to an efficient immune response that manifests as tissue injury. If the available MHC molecules do not present the hapten-modified peptide the modification will remain unnoticed. Also, if the innate immune response isn't activated, the modification may be ignored which means there will be no effector immune response (Pichler, 2013). Moreover, many reactive compounds bind to proteins and modify cell-signalling pathways without causing an immune response. In fact, if the level of modification exceeds a threshold, cells are directed to die by apoptosis, preventing the release of its cellular content and the pro-inflammatory mediators (Behrens et al., 2007).

Some drugs are not reactive by themselves but they undergo metabolism (bioactivation) to produce reactive metabolites, which then can bind to the proteins to induce an immune response. These drugs are called **pro-haptens** e.g. sulfonamides do not bind directly to protein but they are metabolized to aa hydroxylamine-metabolite by cytochrome P450 enzymes and peroxidases (Cribb and Spielberg, 1992). The hydroxylamine undergoes spontaneous oxidation to yield nitroso-sulfamethoxazole (SMX-NO) (Naisbitt et al., 1999) a hapten that binds irreversibly to cellular and serum protein (Naisbitt et al., 2002)(Callan et al., 2009). Moreover, the adducts are immunogenic and antibodies that react with sulfonamide-modified protein have been detected (Harle et al., 1988). T-cells that are isolated from hypersensitive patients have been shown to secrete cytokines

and kill target cells in response to sulfonamide metabolite challenge (Nassif et al., 2002).

Although the main site of bioactivation is the liver by the action of cytochrome P450 in hepatocytes, the result of such bioactivation frequently is tolerance due to the tolerogenic behaviour of liver immune cells (Crispe, 2003). Otherwise, if the drug escapes liver environment and is metabolized in other tissues e.g. keratinocytes of the skin, it may lead to cutaneous hypersensitivity reactions with or without systemic symptoms (e.g. drug rash with eosinophilia and systemic symptoms [DRESS]) (Bowen et al., 2004).

In contrast, it has been proposed that (1) hydroxylamine formation occurs in the liver and (2) the hydroxylamine circulates in the body and undergoes oxidation in organs such as the kidney giving rise to isolated interstitial nephritis (Spanou et al., 2006).

Inactivation of drug metabolites usually follows bioactivation and the balance between the two processes is affected by many genetic and environmental factors which increase or decrease the level of reactive metabolites individuals are exposed to (Posadas and Pichler, 2007). It is important to note that not all metabolites are protein reactive. If they are not transformed into haptens, they may trigger the immune system in a way similar to that of the parent drug e.g. p-i concept *discussed in detail below with allopurinol/oxypurinol* (Pichler, 2013).

Metabolic activation constitutes the base upon which the **prohaptten hypothesis** stands; however, to date, no one can draw a detailed picture which shows a direct correlation between drug bioactivation in vitro and immunological ADRs in clinical practice. This is due to the fact that not all the drugs that are bioactivated will cause hypersensitivity reactions; Moreover, not all drug hypersensitivity reactions necessitates drug bioactivation as an essential step (Park et al., 2010).

- **The danger signal and its relation with the hapten and pro-hapten hypothesis**

Ongoing research has shown a weak point in the hapten and the prohaptens hypothesis regarding its consistency. The following question has been raised, “If a specific drug forms reactive metabolites, why it does not always produce a hypersensitivity reaction?”

This issue was the basis for the Danger Hypothesis proposed by Matzinger (Matzinger, 1994) and discussed in the context of drug hypersensitivity by Pirmohamed (2002) and Uetrecht (1999). According to this hypothesis, the immune response that is produced by the hapten/prohaptens-protein adduct should be strengthened by a signal (danger signal), otherwise the immune response will wean off giving rise to tolerance instead of an immune reaction.

This hypothesis suggests that immune reaction is the product of three elementary signals outlined by (Curtis et al., 1999).

Signal 1: is the net result of the interaction between antigen presenting cell (APC) MHC class I molecules and the TCR on T-cells. Being devoid of any other signals anergy will ensue.

Signal 2: is the net result of co-stimulatory and co-inhibitory molecule interaction between APCs and the T-cells. Costimulatory molecules are ignited when APCs are activated by danger signals that are generated from stressed cells (Pichler et al., 2010). Such an interaction would shape the outcome of the T-cell mediated response that would propagate into tissue injury and a hypersensitivity reaction (Sckisel et al., 2015; Schwartz, 2003) (**Figure 1.2. & Table 1.2**).

Table 1.2: The main co-stimulatory and co-inhibitory molecules. The interaction of these molecules between APCs and T-cells shape the outcome of the T-cell immune response.			
Co-stimulation		Co-inhibition	
APC	T-cell	APC	T-cell
CD40	CD40L	PD1-L	PD-1
ICOS-L	ICOS	CD80/86	CTLA4
CD80/86	CD28	HVEM	BTLA

Signal 3: is polarizing cytokines, which polarize T-cells to either a Th1 or Th2 phenotype. Th1 secreting cells are known for their pivotal role in stimulating macrophages and triggering cell mediated immunity by the release of IL-12 and IFN- γ . On the other hand, Th2 cells have a fundamental role in humeral immunity through the release of IL-4 and IL-13 which mediate immunoglobulin class switching from IgG to IgE (Naisbitt et al., 2000).

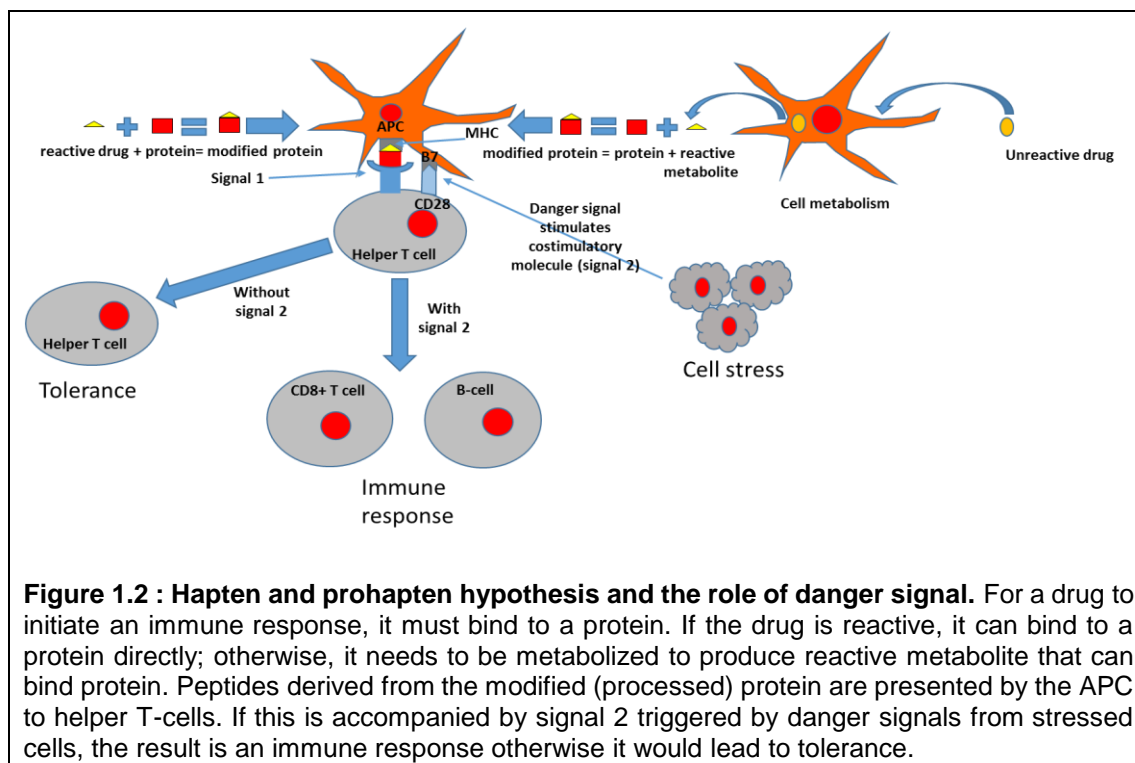


Figure 1.2 : Hapten and prohapten hypothesis and the role of danger signal. For a drug to initiate an immune response, it must bind to a protein. If the drug is reactive, it can bind to a protein directly; otherwise, it needs to be metabolized to produce reactive metabolite that can bind protein. Peptides derived from the modified (processed) protein are presented by the APC to helper T-cells. If this is accompanied by signal 2 triggered by danger signals from stressed cells, the result is an immune response otherwise it would lead to tolerance.

The actual nature of the danger signals is presently ill-defined; however, there are now many possible candidates which include interleukins IL-2, IL-1 α , tumor necrosis factor TNF- α , and interferon IFN- γ , high mobility group box 1 protein (HMGB1), heat shock protein (HSP70), S100 proteins and uric acid (Li and Uetrecht, 2010). These molecules share the feature of acting through toll-like receptors. Any kind of cell stress and/or necrotic cell death would trigger the release of these danger signals. Such cell stress could be caused by physical or chemical factors or infection, which may explain the higher incidence of drug hypersensitivity in patients with conditions such as HIV or cystic fibrosis. Another good example for this is the high prevalence of rash 95% in patients who receive amoxicillin in the presence of EBV infection (Sullivan and Shear, 2001). This can be explained by the high level of inflammatory cytokines which mediates the status of tissue stress and augments the potential for drugs to cause hypersensitivity reactions (Pirmohamed and Park, 2001). Thus, in addition to its importance in explaining the drug hypersensitivity, the danger hypothesis provides the answer for the question, “Why drug hypersensitivity is more common in patients with certain infectious diseases?”

Recent studies have shown that polymorphism of the genes controlling the synthesis of these “danger signals” has an important influential role on the occurrence and severity of drug hypersensitivity (Ghosh et al., 2011).

1.4.1.2 The p-i hypothesis (pharmacological interaction)

According to p-i hypothesis chemically inert drugs are not capable of interacting with proteins to form adducts but they interact with TCR and/or MHC molecules (I or II) (**signal 1**) to induce immune cells. This interaction is not of the kind of irreversible covalent binding (as in hapten or prohaptent hypothesis), instead it is a series of reversible Van der Waals interactions, electrostatic forces and/or hydrogen bonds. Therefore, these drugs are less toxic in comparison to drug hapten (Pichler, 2013). The players in p-i interaction are the **drug**, **TCR molecule** ($\alpha\beta$) and **peptide-MHC complex**. MHC molecules include those expressed on

tissue cells and professional antigen presenting cells. In contrast to haptenic reactions involving drugs such as the β -lactam antibiotics, B-cells or NK cells are not activated via a p-i mechanism (Pichler et al., 2015).

For this hypothesis to be effective, several criteria must be met; first; T-cells with specific TCR for the drug must be present. Secondly, such cells should have a low threshold so that they are stimulated by a low binding threshold. Finally, the TCR should interact with MHC on APCs to strengthen the immune response (Ghosh et al., 2011). Recent research suggests that these conditions exist in the skin of susceptible patients. Skin contains huge populations of effector memory T-cells which may act as a sentinel cells (Rogers et al., 2000). Such memory T-cells have a low threshold for activation in comparison to the naive T-cells since they were primed by prior peptide contact. In addition, skin contains a dense network of dendritic cells that can act as APCs.

Clinical features patients with hypersensitivity that support the p-i hypothesis include:

- Positive skin test response to inert drugs in the absence of any known cutaneous metabolism of these drugs
- The immune response is sometimes observed at the first encounter without a latent period for sensitization which indicates that the drug interacts with pre-existing specific memory T-cells.
- Generalized immune responses to drugs occur in the absence of local danger signs.
- The immune response reflects an abnormal vigorous T-cell stimulation with massive destructive (or even fatal) outcome as seen in SJS/TEN and DRESS (Pichler et al., 2010).

A brief comparison of hapten and p-i mechanisms is shown in **Table 1.3**.

P-i reactions are divided into two types according to the preferential binding to either TCR or HLA:

1.4.1.2.1 P-i TCR

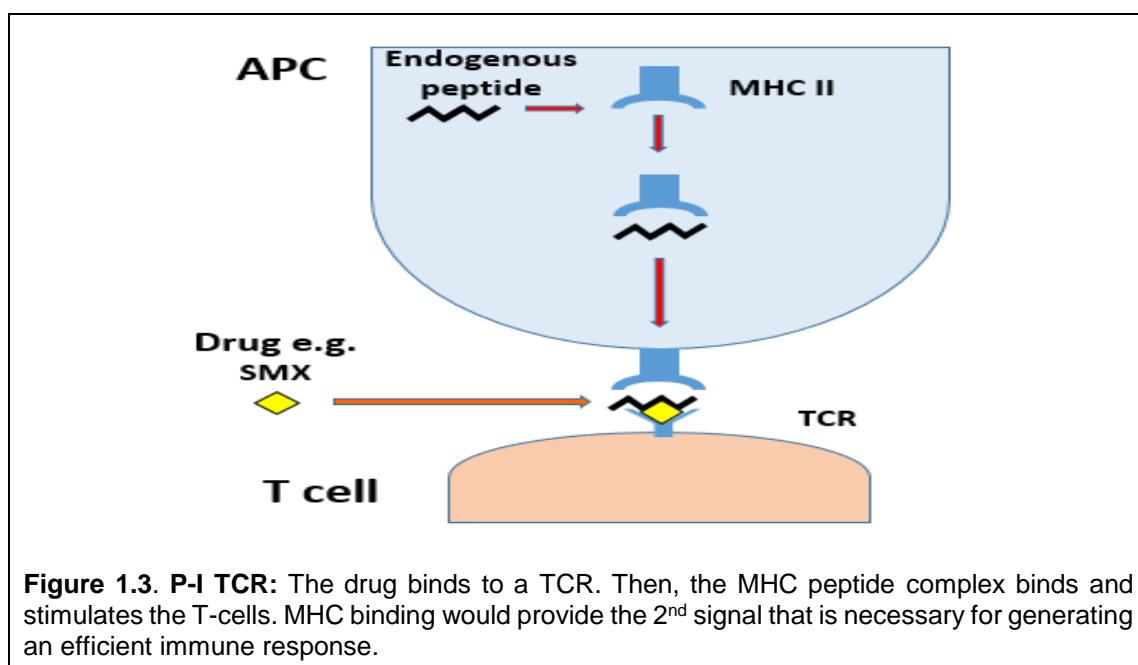
The drug binds primarily to the specific TCR molecule (by non-covalent labile bonds) rendering the interaction of MHC molecule (of APC) a necessary but only an additional stimulatory effect for the development of an efficient immune response (Depta et al., 2004; Schmid et al., 2006) (**Figure 1.3**). This pathway evolved through computer modelling of drug TCR interactions and it had been proposed that CD4+ T-cells clones (TCCs) might be activated via this pathway in an MHC class II restricted fashion (Pichler et al., 2015).

Table 1.3. Comparison of the hapten and p-i concepts adapted from (Pichler et al., 2015)	
Hapten concept	P-i concept
Chemical binding to proteins or peptides which may act as antigens for T and/or B-cells	Structural binding to HLA or TCR leading to direct and exclusive T-cell stimulation. No role of innate immune response.
Covalent interactions	Non covalent interactions (labile)
Often dependent on processing by APC and drug metabolism	Processing and metabolism not required
Onset depends the time required for drug metabolism and processing within APCs (> 4hrs)	Mostly immediate (<10 min) to activate T-cells
Important role for danger signals	Danger signals have no role in p-i HLA but some p-i TCR reactions may require danger signals

Research conducted with lidocaine and the SMX models have shown that the MHC bound peptide could be removed or exchanged without affecting CD4+ T-cell activation (von Greyerz et al., 2001; Hari et al., 2001). Moreover, some clones reacted to the drug in the presence of allogenic MHC alleles indicating the absence of HLA restriction for lidocaine and SMX. (Zanni et al., 1999; von Greyerz et al., 2001).

A recent study has shown that the response of certain SMX specific TCCs can be blocked by structurally related sulfanilamides (Watkins, 2013). Docking studies with these blocking sulfanilamides show that they bind to the same site as SMX (CDR3), but without stimulating T-cell signalling (Pichler, 2013). Why might this be the case? It was found that only SMX binds to CDR3 with its NH_2 group pointing to the peptide-binding groove (Pichler et al., 2015).

In contrast, the NH_2 group of non-stimulatory sulfonamides points toward the TCR (J. Pichler and Watkins, 2014). Thus, it seems that the orientation of bound drug serves a role in the activation of T-cells.



Another example of the vital role of certain TCR clonotypes (clonotype is defined as T-cells that express identical TCR phenotype) is TCR triggering in carbamazepine (CBZ) hypersensitivity. Patients with CBZ-induced SJS/TEN and HLA-B*15:02 background react to normal peptides and not against hapten-modified peptides (Yang et al., 2007). The T-cells use the TCR-V β -11-ISGSY clonotype (Ko et al., 2011). This clonotype was present in 16 of 19 patients and absent in all the 17 CBZ-tolerant patients. Moreover, CBZ-specific T-cells could be primed in vitro using PBMCs of healthy individuals who were carriers of the

HLA risk allele and TCR-V β -11-ISGSY. These data show that in some disease manifestations the TCR phenotype may play a crucial role.

1.4.1.2.2 p-i HLA

One of the important steps toward understanding the mechanisms underlying hypersensitivity reactions was the description of a strikingly high HLA allele association for certain severe hypersensitivity reactions. The HLA allele associations were extremely high with a negative predictive value near 100% for certain severe reactions linked to specific drugs (Hung et al., 2005; Chung et al., 2004; Mallal et al., 2002). This high association raised the question: Is the T-cell response dependent on the drug binding to the allele itself or the presented peptide?

The majority of reported associations were for HLA class I alleles, but some were for HLA class II alleles (Singer et al., 2010) mostly resulting in less severe reactions. There is no role for danger signals in T-cells activation (Pichler et al., 2015).

Mass spectrometry has been used to show that peptides eluted from MHC molecules are not modified covalently with either CBZ or abacavir (Yang et al., 2007; Norcross et al., 2012; Ostrov et al., 2012). The binding interaction of these two drugs with the respective HLA risk alleles (HLA-B*15:02 [in Han Chinese] and HLA-B*57:01) was characterized by crystallography (Chung et al., 2004; Illing et al., 2012). Both drugs bind to the HLA molecule directly underneath the antigenic peptide.

Mechanisms of abacavir-specific T-cell activation has been studied extensively and the results obtained has resulted in new models of drug-specific T-cell activation. Specifically, the drug has been shown to modify HLA-B*57:01 molecules via intracellular and extracellular pathways discussed in detail below: **(Figure 1.4).**

- **The Altered Peptide Model of p-i HLA (intracellular pathway)**

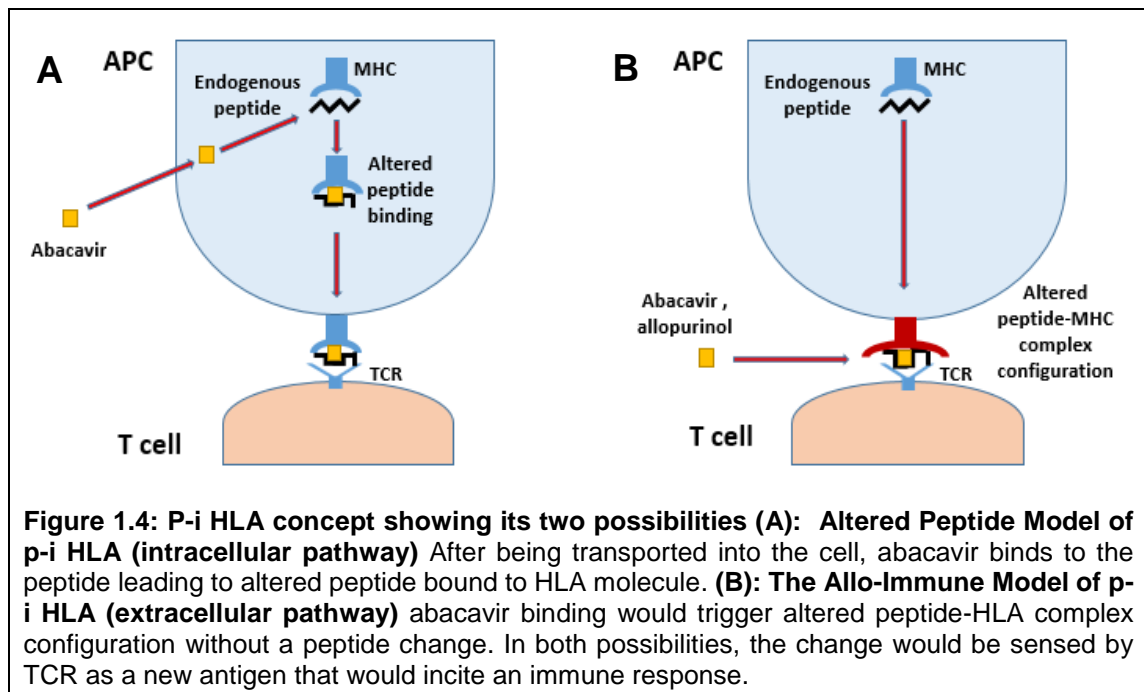
After being transported into the cell, abacavir enters the endoplasmic reticulum; within the endoplasmic reticulum, it competes with peptides for binding with F9 pocket of HLA-B*57:01.

Abacavir alters the structure of HLA-B*57:01 and hence the peptide binding properties of the MHC molecule. Altered peptides then bind to the modified MHC in place of the usual peptides (Illing et al., 2012). Thus, abacavir binding would make the abacavir-HLA-B*57:01 appear like a foreign HLA-protein. Illing et al (2012) proposed that these new abacavir-modified HLA-B*57:01 binding peptides activate T-cells that cause hypersensitivity in the exposed patients; however, the nature of these peptides is yet to be defined.

- **The Allo-Immune Model of p-i HLA (extracellular pathway)**

By the extracellular pathway, abacavir binds directly to F9 pocket of the peptHLA-B*57:01 complexes (Pichler et al., 2015).

Experiments using abacavir and allopurinol suggest that drug binding immediately changes the peptHLA complex configuration without a peptide change. Such a binding interaction is thought to trigger direct allorecognition by T-cells (Pichler et al., 2015). Drug binding occurs due to the flexibility of the peptHLA binding which allows abacavir to enter the binding site below the peptide without the need for peptide exchange (Yun et al., 2014).



1.4.2 Role of costimulation (danger signals) in p-i reactions

Costimulation is not required in p-i HLA abacavir model, since abacavir-B*57:01 peptide complexes are thought to act as an allo-allele and incite a strong direct T-cell response.

On the other hand, p-i TCR reactions are different and some may require costimulation. There are two possibilities:

(1) The presence of a high-risk allele could be relevant when a peculiar T-cell clonotype is present (as the activation of CBZ specific T-cells in the presence of HLA-B*15:02 and TCR-V β -11-ISGSY).

(2) Activation of T-cells by concomitant infection yielding upregulation of adhesion molecules and cytokine secretion. This may lower the threshold for T-cell reactivity (Pichler et al., 2015).

1.4.3 Which hypothesis is the predominant? Hapten or Pharmacological Interaction?

Advocates of the hapten hypothesis have used the sulfamethoxazole (SMX)-nitroso (which is a metabolic derivative of SMX) as an example to support their hypothesis. SMX-nitroso acts as a hapten to instigate antigen-specific T-cell responses. SMX-nitroso is deactivated in vivo by antioxidants such as the non-protein thiol glutathione (or ascorbate) and the addition of glutathione to T-cell assays blocks responses to SMX-nitroso. Individuals with low levels of cellular thiols (like people suffering from HIV) have higher levels of SMX-nitroso in their body. Patients with HIV develop hypersensitivity to SMX more often than the normal population and this has been attributed to decreased detoxification of the nitroso metabolite (Naisbitt et al., 2000).

On the other hand, it has been shown that the addition of glutathione does not inhibit T-cell responses to the parent drug (SMX). In fact, clones responsive to the parent drug respond vigorously in the presence of SMX-nitroso and glutathione. This finding is explained by the reduction of the nitroso metabolite back to the parent drug and the activation of T-cells via the pharmacological interaction hypothesis (Naisbitt et al., 1999;Burkhart et al., 2001).

1.4.3.1 Other mechanistic hypotheses

In addition to the above mentioned mechanisms, the immune system can also be triggered by alteration in the immune balance , epigenetic effects and direct activation of antigen presenting cells (Zhang et al., 2011).

1.4.3.1.1 The immune imbalance

The immune system is highly regulated, and various mechanisms control the balance between activation and tolerance. Over recent years, many new biological drugs have been developed to control autoimmune diseases such as multiple sclerosis (Uetrecht, 2009).

However, unexpectedly, although these drugs were developed to regulate immunosuppression, the response in some patients was autoimmunity. For example, anti-TNF antibodies have a promoter effect for a variety of immunological disorders like vasculitis and lupus (Ramos-Casals et al., 2010; Mielke et al., 2008; Gonzalez-Lopez et al., 2008). Similarly, an interleukin-1 receptor antagonist (Anakinra) induce psoriasis (Mielke et al., 2008; Gonzalez-Lopez et al., 2008). Although the mechanisms underlying these findings are not clear, they may be linked to an unexpected alteration in the balance between activation and tolerance (Uetrecht, 2009). For example, anti TNF antibodies cause a shift of the T helper profile through blocking Th1 cytokine. Consequently, there would be a shift of the immune system toward Th2 profile. This would lead to the production of autoantibodies that would mediate the lupus like syndrome (Dedeoglu, 2009).

1.4.3.1.2 Epigenetic effects

Epigenetic effects are related to changes in gene expression that are caused by modifications of chromatin proteins (e.g. histone acetylation) , DNA (cytosine 5-demethylation) (Szyf, 2009) and distinctive RNA types (e.g. non-coding RNA, miRNA and mRNA) (Trerotola et al., 2015). It has been shown that the pathogenesis of cancer, asthma, and some autoimmune diseases is linked to epigenetic changes. For example, hypomethylation of DNA in T-cells is related to the development of lupus disease. Epigenetic effects may be a potential mechanism involved in the generation of IDRs (Tesfa et al., 2009). For example, azacytidine, which is an anticancer drug used in treatment of myelodysplastic syndromes is also a causative factor for neutropenia via DNA hypomethylation-induced apoptosis (Khan et al., 2008).

1.4.3.1.3 Direct activation of antigen presenting cells

Many drugs undergo oxidation to reactive metabolites by the action of myeloperoxidase in neutrophils and the antigen presenting cells (Uetrecht, 1990).

Such reactive metabolites stress APC or bystander cells and cause activation of APCs (i.e., upregulation of costimulatory receptors and/or cytokine release (Sanderson et al., 2007).

The interaction between the APCs and the T-cell involves forming a reversible imine bond between the aldehyde group on the APC and an amino group on the T-cell (Rhodes, 1996). Drugs that have a hydrazine group in their structure like penicillamine and isoniazid bind to the aldehyde group on APCs irreversibly triggering their activation. This mechanism might contribute to the IDRs seen in patients exposed to isoniazid and penicillamine (Li et al., 2009; Rhodes, 1996).

1.4.3.1.4 Bystander activation

Bystander activation is the term given to the activation of unrelated (heterologous) T-cells with a different antigen specificity during an antigen specific T-cell response. This results in the production of phenotypic or functional changes through mechanisms that are independent of specific TCR stimulation. Bystander activation is thought to involve membrane bound receptors or soluble factors that bind to receptors other than TCRs (Bangs et al., 2006; Boyman, 2010).

Bystander activation has been described in detail for CD8⁺ T-cells. Less is known about the bystander activation of CD4⁺ T-cells.

Danger signalling molecules like LPS causes upregulation of activation markers in naïve and memory T-cells (Kamath et al., 2005). Furthermore, IFN- α/β induces memory CD8⁺/CD4⁺ T-cells proliferation that is mediated through IL-15 production (Tough et al., 1996). Naïve CD8⁺ T-cells need an additional requirement that is the presence of MHC molecules in which there is “tickling” of TCR by interaction with low affinity self-peptide-MHC complexes without specific TCR stimulation. Following this activation naïve T-cells acquire memory phenotype (Kieper and Jameson, 1999).

An alternative pathway has been suggested by Di Genova et al. for activation of heterologous memory CD4⁺ T-cells which express high levels of IL-2R α CD25

(CD25^{high}) and CD122^{int}. These cells are proposed to undergo bystander activation by responding to IL-2 secreted from Ag-specific CD4⁺ T-cells stimulated by the TCR interacting with the MHC II-specific antigen complex (Di Genova et al., 2010).

Bystander activation may play a role in drug hypersensitivity via two mechanisms:

1. The activated DCs or the activated specific CD4⁺ T-cells by the drug antigenic determinants (which could be drug-modified peptides) would activate other (heterologous CD4/CD8) T-cells which are specific to alternative antigenic determinants that are present at sub-threshold levels. This would lead to an augmented drug-specific T-cell response and a more severe clinical syndrome.
2. The activated DCs or the activated specific CD4⁺ T-cells by the drug antigenic determinants would activate the other (heterologous CD4/CD8) T-cells which are specific to autoantigens leading to a plethora of drug hypersensitivity reactions.

1.4.3.1.5 Extended antigen priming (EAP)

Extended antigen priming is defined as T-cell activation in response to suboptimal doses of cognate antigen x in the presence of fully activated T-cells in response to optimal dose of different antigen y. So far, this phenomenon has only been proven for CD4⁺ T-cells.

This theory was proposed after the study of a three cell model incorporating DCs, responder T-cells specific to antigen y and an associator T-cell specific to antigen x. All these cells were in an environment in which there is optimal doses of antigen y and suboptimal doses of antigen x. The associator cells were shown to have a significantly higher proliferative response to suboptimal doses of antigen x, than when incubated with antigen x alone (Shibuya et al., 2015).

The response was independent of IL-2. This represents a distinctive feature that differentiates EAP from bystander activation of CD4⁺ T-cells, which is highly

dependent on IL-2 (Di Genova et al., 2010). EAP is however, dependent on CD40-CD40L interaction which mediates costimulatory signals for CD4+ T-cell proliferation and IFN- γ secretion (Whitmire et al., 1999). It is postulated that responder cells stimulate the associator T-cells by providing critical signals like IL-12, IL-18, and IL-15 (Sattler et al., 2009). Moreover, the danger signals such as TLRs stimuli for APCs may trigger EAP by enhancing the secretion of costimulatory molecules and cytokines (Shibuya et al., 2015).

1.4.4 Risk factors for drug allergy:

1.4.4.1 Drug factors:

- **Nature of the drug:**

In general when the drug is highly protein reactive in nature, a high incidence of sensitization is expected (Thong and Vervloet, 2014). Moreover, the molecular weight of the drug may determine the mechanism of T-cell activation and the subsequent hypersensitivity reaction (see *the mechanisms of drug allergy*).

- **Degree of exposure (dose, duration, frequency):**

Sensitization is more frequent with higher doses and prolonged administration. Of great importance is intermittent administration in comparison to prolonged use. An example of this is penicillin, which has a high incidence of allergic reactions in the first two weeks of repeated administration compared to prolonged use (Thong and Vervloet, 2014).

- **Route of Administration:**

Topical administration is associated with the highest rates of cutaneous sensitization and should be avoided with certain agents. The intravenous route may be the least sensitizing route although it may be associated with severe anaphylactic reactions (Thong and Vervloet, 2014).

1.4.4.2 Host Factors

- **Age and Sex:**

Some allergic reactions are less frequent in children and elderly people possibly owing to the immaturity or involution of the immune system respectively. Moreover, children are not generally exposed to multiple courses of the same drug. Allergic drug reactions are more common in females than males (Thong and Vervloet, 2014).

- **Pharmacogenetics** (e.g. HLA type, acetylator status):

Acetylation is an important metabolic detoxifying mechanism for drugs such as sulfonamides, isoniazid and hydralazine. The slow acetylator phenotype (common in African and Caucasian individuals (50-65%)), leads to drug accumulation in the body with its subsequent side effects (Scott and Thompson, 2011).

The discovery of a surprisingly strong association between expression of HLA alleles and specific forms of drug-induced liver and skin injury has changed the way in which researchers view this form of iatrogenic disease. In fact, certain reactions are no longer completely unpredictable. An example for this is the HLA-B*1502 associated with carbamazepine induced SJS/TEN in Han Chinese in Taiwan, Thailand and India. However, some of these HLA allele associations are only seen in certain ethnicities. For example, HLA-B*1502 is not associated with carbamazepine induced SJS/TEN in patients with either Japanese or Europeans of non-Asian ancestry (Chung et al., 2004).

Atopy by itself is not a risk factor for the development of allergic drug reactions although asthmatic patients are more prone to have severe reactions (as in food allergy) (Kewalramani and Bollinger, 2010).

- **Concurrent medical illness:** (e.g. Epstein Barr virus (EBV), human immunodeficiency virus (HIV)).

The disease may promote the development of allergic drug reactions by altering the metabolic pathways and the immune status of the patient. Certain infections are associated with increased likelihood of developing an allergic drug reaction e.g. ampicillin maculopapular rashes in the patients with EBV (Epstein Barr virus) infections (Sullivan and Shear, 2001) and co-trimoxazole hypersensitivity in patients with HIV or AIDS (Harb and Jacobson, 1993).

- **Multiple Drug Hypersensitivity Syndrome:**

Certain patients will develop allergic reactions to multiple drugs. This phenomenon is referred to as multiple drug hypersensitivity syndrome (Thong and Vervloet, 2014). In recent years, Daubner et al (2010) reported that drug-reactive T-cells in patients with multiple drug hypersensitivity are contained in an in vivo pre-activated T-cell fraction and show a lower threshold for activation by drugs. Moreover, the patients have been shown to develop a primary immune response against each drug as studies with cloned T-cells revealed an absence of crossreactivity (Pichler et al., 2011).

1.4.5 Genetic background of patients with drug hypersensitivity reactions

There has been renewed interest in Pharmacogenetics after the completion of the first draft of the human genome. It is now recognized that most drug hypersensitivity reactions are polygenic. The outcome of drug exposure is dependent on the interaction of multiple genes in addition to the presence of specific, as yet ill-defined environmental factors. Each gene increases the risk of developing hypersensitivity reactions nevertheless it cannot per se induce the reaction.

Over recent years, many genes have been shown to be risk factors for hypersensitivity reactions. Overall, these genes can be divided into four major categories:

1.4.5.1 Bio-activation genes

The genes control drug metabolism leading to the production of stable or reactive metabolites. This process is achieved by phase I drug metabolizing enzymes, which mediate oxidation, reduction and hydrolysis reactions (Bugelski, 2005). The most important of these are the cytochrome P450 superfamily of enzymes. Many of which are expressed polymorphically.

1.4.5.2 Bio-inactivation genes

These are genes that influence the bioinactivation of the drug metabolites. Bio-inactivation genes encode phase II enzymes that catalyse acetylation, sulfation, glutathione and glucuronidation reactions. The phase II metabolizing enzymes enhance the rate of detoxification of drugs. Altered detoxification can result in the development of immunogenicity. An example for that is the thiomersal toxicity (Bugelski, 2005).

1.4.5.3 Genes controlling immune-responsiveness

Genes coding for MHC, T-cell receptors, and co-stimulatory/co-inhibitory molecules control the generation of the principal mediators of the cellular immune response. These are discussed in much greater detail below.

1.4.5.4 Genes controlling tissue injury and repair

The balance between these genes is of critical importance for limiting tissue damage. Typically, the candidates would be cytokines, chemokines and prostaglandins.

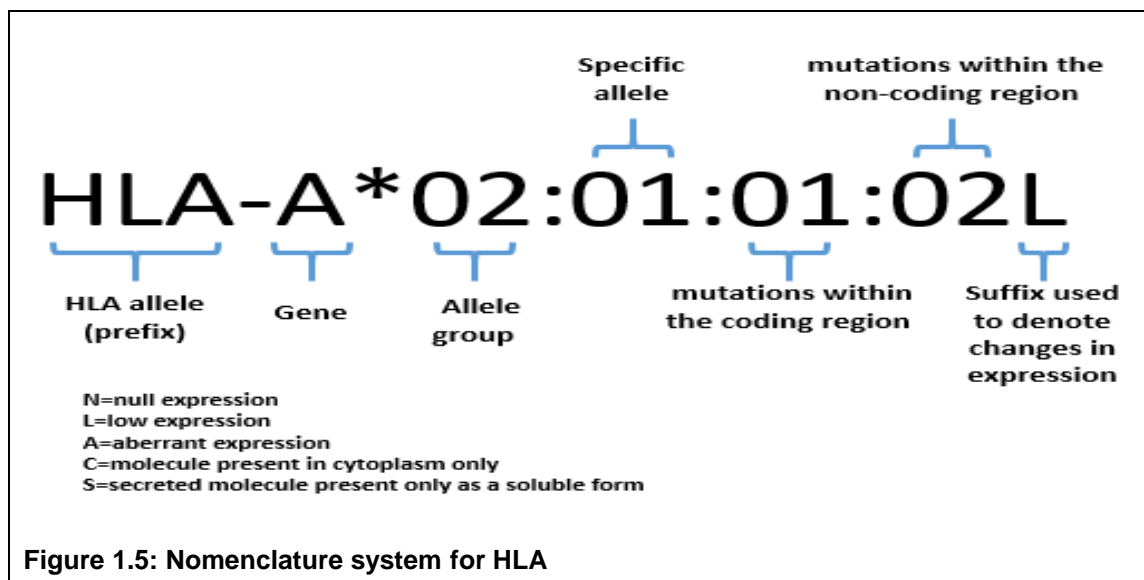
Although all the previously mentioned classes of genes are necessary to trigger an immune response to a drug, the latter two types are by far the most influential (Naisbitt et al., 2003a).

1.4.6 HLA typing, the old suspect, the new method

HLA (human leukocyte antigen) system is a gene complex on chromosome 6 that is responsible for the expression of two types of MHC molecule: MHC-I (includes MHC-A, B and C) which are expressed by all nucleated cells, MHC-II (includes MHC-DP, DQ and DR) which are expressed by APCs, some endothelial cells, thymic epithelial cells and B-cells.

Each individual has two sets of six HLA allele genes. **Figure 1.5** summarizes the system for naming HLA alleles.

MHC molecules have a vital role in adaptive immunity by presenting antigens to TCRs of T-cells. Since the discovery of HLA is a growing evidence of a relationship between expression of specific HLA alleles and susceptibility to different forms of drug hypersensitivity reaction.



Earlier studies utilized serology for HLA typing. In contrast, nowadays high-resolution genotyping has been introduced. The knowledge of linkage disequilibrium patterns has made a significant contribution by helping researchers

to reach a definitive conclusion particularly in relation to the anti-retroviral drug, abacavir induced SJS with HLA-B*57:01 (Mallal et al., 2002); antiepileptic drugs (AEDs): carbamazepine (CBZ) and phenytoin (PHT) (Locharernkul et al., 2008) and lamotrigine (LTG) induced SJS with HLA-B*15:02 in Han Chinese (Man et al., 2007) and allopurinol induced SJS in Han Chinese with HLA-B*58:01 (Hung et al., 2005).

Carbamazepine (CBZ) contains an aromatic ring, which is shared between several drugs. These include phenytoin (PHT), lamotrigine (LTG) and oxcarbazepine (OXC). Clinically, it is known that certain patients exposed to each of these drugs develop a multiple hypersensitivity syndrome. A study conducted in Thailand has shown that the presence of HLA-B*15:02 allele is a risk factor for developing hypersensitivity to PHT in addition to CBZ. Consequently, HLA-B*15:02 has gained extra focus by finding that it confers a class effect in addition to conferring a drug effect. Such a discovery has paved the way for the recommendation to avoid CBZ, OXC and PHT for the HLA-B*15:02 carrier, while LTG should be used with caution (Hung et al., 2010).

- **Genetic predisposition is specific for the phenotype**

It should be kept in mind that the association of HLA-B*1502 allele is only valid for CBZ-induced SJS/TEN, but not for other types of CBZ hypersensitivity like maculopapular reactions or DRESS (Li and Uetrecht, 2009, Pichler, 2003). These conditions are associated with HLA-A*31:01 carriage in individuals of European and Japanese ancestry.

HLA-B*1502 is more prevalent in Han Chinese while HLA-A*3101 is more prevalent in Europeans and Japanese ancestry. Consequently, the CBZ-induced reactions profile varies with location (i.e., SJS and TEN are more common in Thailand) (Hsiao et al., 2014).

1.4.7 Clinical features of drug hypersensitivity

It is important to realize that most immune responses to drugs are silent, i.e. in most cases of contact with the drug under investigation there is no overt clinical presentation of drug hypersensitivity. In patients that develop clinical manifestations, diagnosis is dependent on several clinical features that suggest an immunological event:

1. Wide spectrum of clinical presentation in terms of both magnitude and nature of the tissue injury to the same drug (**Table 1.4**) (Bugelski, 2005). The famous example is halothane, which can cause two forms of drug-specific hepatic damage. Reductive metabolism leads to a mild form of self-limiting tissue injury referred to as type I hepatitis (in up to 20% of patients). On the contrary, oxidative metabolism may result in the production of acyl halide metabolites that bind to protein and lead to the development of immune-mediated hepatotoxicity (type II hepatitis). Although the latter is less common than type I hepatitis, it is more severe and has a higher mortality (Naisbitt, Pirmohamed and Park, 2003)
2. Constitutional symptoms of fever, rash, eosinophilia, arthralgia and lymphadenopathy.
3. Latency period of about two weeks for the reactions to manifest.
4. On re-exposure to the provoking agent, the reactions appear more rapidly and are more severe.
5. Resolution of the reactions after drug withdrawal. Moreover, reactions can be averted by the gradual increase in the dose over time; a process that indicates the development of tolerance (Naisbitt et al., 2003a).

Table 1.4. Expression of Immune Mediated Adverse Effects. Adapted from (Bugelski, 2005)			
Severity of Response	Effector Mechanisms	Clinical presentation	Examples
No overt signs or symptoms	Cell mediated Humoral	In vitro proliferation of lymphocytes antidrug antibodies	Sulfamethoxazole ,Nomifensin and amodiaquin
Trivial or mild	Cell mediated Humoral	Maculopapular exanthema, Urticaria	Amoxicillin Cephalosporins
Moderate	Cell mediated Humoral	Bullous exanthema, Anticonvulsant hypersensitivity syndrome	Amoxicillin Phenytoin
Severe	Cell mediated Humoral	Epidermal necrolysis Haemolytic anemia	Sulfamethoxazole Penicillin
Life threatening	Cell mediated Humoral	Hepatic failure Anaphylaxis,	Halothane Penicillin

1.4.8 Clinical presentation of IDRs:

1.4.8.1 Autoimmunity:

Autoimmunity is the inability of an organism to identify its constituent parts as self. This results in the action of its own immune system against these cells or tissues. Autoimmunity involves the development of autoantibodies and/or effector T-cells with specificity against self-proteins. The clinical presentation is broad and can be classified into either a generalized autoimmune reaction (e.g. idiopathic lupus) or organ specific reactions such as auto immune hemolytic anemia, autoimmune hepatitis and pemphigus vulgaris (Pichler, 2003).

Accordingly, the autoantibodies may develop against the nuclear antigen, erythrocytes or other protein antigens. These autoantibodies do not disappear immediately after withdrawal of the drug but they take several weeks to do so since the autoantigen is still present. Many drugs are known to produce

generalized autoimmune reactions (Fritzler and Tan, 1978). However, the clinical and the laboratory features of these reactions overlap with the idiopathic form so after drug withdrawal it is hard to differentiate between them (Wiik, 2008). Common clinical presentations of drug-induced lupus include fever, myalgias, arthritis, and serositis involving the pleura and/or the pericardium. Drugs known to induce lupus include biological agents such as anti-TNF- α , (IFN)- α and various cytokines. Autoimmunity often develops around one year after starting treatment in spite of the fact that antibodies and specific T-cells are detectable much earlier (Woosley et al., 1978). The detection of an antigen-specific response ex vivo is an essential characteristic for the diagnosis (Zhang et al., 2011).

1.4.8.2 Skin rashes

These are the most common clinical feature of drug hypersensitivity. In contrast, other reactions like allergic hepatitis, nephritis and agranulocytosis occur much less frequently (Bugelski, 2005).

The skin rashes induced by drugs ranging in severity from the mild maculopapular rash to life-threatening Steven Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN).

1.4.8.2.1 Drug induced urticaria

It represents only 5% of all cutaneous drug reactions (Nigen et al., 2003). Urticarial lesions also known as “hives” are itchy, red, blotches or wheals, which are pale in the centre while red around the rim. They are widely distributed over the body and sometimes associated with swelling of the subcutaneous tissues known as “angioedema”. These lesions disappear within few hours without a remnant trace. β -lactam antibiotics are common causes of urticaria. Binding of the β -lactam ring to proteins and the production results in immune activation and the generation of IgE antibodies with specificity against the drug modified proteins (Levine and Price, 1964). It should be kept in mind that not all drug-induced urticarias are caused by antibodies. For example, the inhibition of kinin

degradation that is caused by angiotensin converting enzyme inhibitors, the altered arachidonic acid metabolism caused by aspirin and other NSAIDs (Mathelier-Fusade, 2006). These are two examples of pseudoallergic (non-allergic) reactions.

1.4.8.2.2 Maculopapular drug eruptions

Maculopapular eruptions are the most common IDRs. Usually these reactions develop within 1-2 weeks after drug exposure and more rapidly on rechallenge or if the patient is previously sensitized (Torres et al., 2009). Maculopapular rashes are T-cell-mediated. Histological analyses have shown a cellular perivascular infiltration of T-cells (mainly CD4) in the dermis together with the expression of skin homing receptors like cutaneous lymphocyte antigen (CLA), the chemokine receptors like CCR6 and CCR10 and an elevated expression of their ligands (CCL20 and CCL27 respectively) in skin. T-cells secrete cytokines and cytolytic molecules when activated with the drug and chemokines that attract other cells inflammatory cells that contribute towards the tissue injury.

1.4.8.2.3 Fixed Drug Eruption (FDE)

Fixed drug eruptions are characterized by the development of erythematous round lesions with brown to dusky purple or red colour plaques and sometimes feature blisters, which develop days or weeks after taking the causative drug. These lesions are associated with itch and/or burning sensation in the affected area. Usually specific areas of the skin are affected. These include the feet, hands and mucosal surfaces. The site of the eruption is fixed, which means that the patient develops the same eruption at the same site, after re-exposure to the drug. The eruption heals 7-10 days after drug withdrawal; however, hyperpigmentation may be permanent (Pal et al., 2014). It is believed that the FDE is mediated by intraepidermal resident memory effector CD8+ T-cells. Weak HLA associations have been described with some drugs such as sulfonamides, tetracyclines and

NSAIDs (Pavlos et al., 2012). Patch test has been reported to be +ve at the site of the lesion but reliability of the assay is drug dependent (Andrade et al., 2011).

1.4.8.3 Drug reaction with eosinophilia and systemic symptoms (DRESS)

The DRESS syndrome is characterized by the development of fever with a widespread maculopapular rash and multi-organ involvement. Furthermore, white cell abnormalities such as eosinophilia are common features of DRESS. Lymphadenopathy is common and often misdiagnosed as viral infection.

80% of cases with DRESS affect the liver, 40% kidneys and 38 % lungs. The heart and pancreas are involved less commonly (Walsh and Creamer, 2011). Research has shown some association with the reactivation of human herpes viruses especially HHV-6 and cytomegalovirus (CMV). DRESS features can occur 2-6 weeks after the discontinuation of the culprit drug. It has been hypothesized that specific-specific T-cells are the initial mediators of the syndrome. Viral-specific T-cells are activated at a later time-point and are thought to be responsible for extending the duration of the disease (Choudhary et al., 2013). Drugs commonly associated with DRESS include carbamazepine, phenobarbital, phenytoin and sulfonamides (Seishima et al., 2006). Laboratory findings show antithyroglobulin and antithyroid peroxidase antibodies in 30-40% of patients (Gupta et al., 1992). In addition to hematological abnormalities in the form of eosinophilia, thrombocytopenia, and atypical lymphocytosis. Reactivation of HHV-6 has been added to the scoring criteria of screening for DRESS in Japan (Shiohara et al., 2006). DRESS has a 10% mortality rate (Walsh and Creamer, 2011).

1.4.8.3.1 Steven Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN)

These are life threatening skin rashes characterized by the development of fever, keratinocyte apoptosis and prominent blistering of the skin. The two conditions

are thought to be mediated via the same immunological mechanism; and the main different being the disease severity. SJS is associated with epidermal detachment of up to 10% of the skin surface area, while in TEN more than 30% skin detachment is observed. Mortality rates in patients with TEN exceed 30%. Detachment of 10-30% of the skin is referred to as transitional SJS-TEN (Mockenhaupt, 2009). The pathology of both SJS and TEN is characterized by extensive keratinocyte apoptosis resulting in the separation of the dermis from the epidermis, which is thought to be caused by T-cells. One possible mechanism involves the interaction of Fas ligand with its receptor on target cells. Another possible mechanism involves cytolytic molecules such as perforin and granzyme B that are released by T-cells. Most recently, granulysin has been found in high levels in blister fluid of patients with TEN indicating that it may be an important mediator of tissue injury (Chung et al., 2008).

1.4.8.4 Hematologic Idiosyncratic Adverse Reactions

1.4.8.4.1 Drug induced hemolytic anemia

Is characterized by increased red cell destruction through the effect of antibody mediated complement activation (Aster, 2010).

One of the famous drugs known to be associated with the production of antibodies is penicillin especially if it is given in high doses for more than 10 days (Garratty, 1993).

1.4.8.4.2 Drug induced thrombocytopenia

Thrombocytopenia occurs when the platelet count is lower than (150000 platelets/ μ L). When the count drops to less than (10000 platelet/ μ L) the patient is at high risk of life-threatening hemorrhage (Aster, 2010). The commonest manifestation of thrombocytopenia is spontaneous bruising, which is usually present after a week or more of the offending treatment (Aster and Bougie, 2007). A famous example of the drug-induced thrombocytopenia is heparin. Antibodies

against the heparin–platelet factor 4 complex have been detected in patients with thrombocytopenia (Warkentin, 2003)(Warkentin, 2003). Although these reactions are immune mediated, they are not necessarily associated with immune memory which is a common feature of immune-mediated reactions (Zhang et al., 2011). Therefore, if a patient with a known history for heparin-induced thrombocytopenia is rechallenged with heparin he may not essentially develop thrombocytopenia or even if he did so it does not occur more rapidly (Warkentin and Kelton, 2001).

1.4.8.4.3 Drug induced agranulocytosis

Agranulocytosis is defined as a neutrophil count less than 500 neutrophils/ μ L of blood. This makes the patient a high risk of acquiring a serious and potentially fatal infection. Agranulocytosis is induced by a large group of drugs including analgesics, antipsychotics, antithyroid medications and anticonvulsants (Andres et al., 2006). Cancer chemotherapy is also a well-known cause of agranulocytosis, but it is usually not idiosyncratic. The drugs mentioned above do cause idiosyncratic agranulocytosis and in several cases the reactions are believed to be immune mediated (Tesfa et al., 2009).

Clozapine induced agranulocytosis is the most widely studied form of agranulocytosis. A weekly blood count is performed in patients receiving the drug to prevent agranulocytosis. Drug-dependent antibodies have not been detected in patients on clozapine therapy. However, clozapine induced neutropenia is related to neutrophil metabolism. Clozapine is oxidized by myeloperoxidase to a nitrenium ion metabolite that binds to neutrophils causing activation of the apoptotic cascade (Williams et al., 2000).

The covalent adducts formed in neutrophils have the potential to initiate an immune response. One study has shown that there is a weak association between clozapine induced agranulocytosis and the presence of certain HLA genotypes in Ashkenazi Jewish population. These HLA genotypes include DRB1*04:02, DQB1*03:02, and DQA1*03:01. While in non in non-Jewish patients there was an

association with HLA-DR*02, DQB1*05:02, DQA1*01:02 (Yunis et al., 1995) , HLA-DQB1 (126Q) and an amino acid change in the extracellular binding pocket of HLA-B (158T) (Goldstein et al., 2014). The time needed to elicit clozapine agranulocytosis is relatively short 6-12 weeks and this period does not shorten on rechallenge so this may suggest an autoimmune mechanism (Uetrecht, 2009).

1.4.8.4.4 Drug induced aplastic anemia

Aplastic anemia is relatively rare in comparison to drug induced agranulocytosis, but its severity had limited the use of several drugs including chloramphenicol and felbamate (Young et al., 2008). It has been suggested that cytotoxic T lymphocytes cause bone marrow destruction. The dramatic response seen in patients exposed to immuno-suppressive therapy suggests that the etiology is immune mediated (Young, 2002).

1.4.8.5 Idiosyncratic liver toxicity

The liver is a major site for the drug metabolism. Realising that IDRs may involve a role mediated by reactive metabolites, it is not surprising that the liver is the target of many reactions. In USA, it has been reported that idiosyncratic drug induced liver injury (IDILI) accounts for about 13% of acute liver failure cases (Ostapowicz et al., 2002). Moreover, it is the most common cause of drug withdrawals from the market (Temple and Himmel, 2002). The mechanisms of IDILI are not well understood and the immune system involvement is more controversial than other types of IDRs (Zhang et al., 2011).

In comparison to other types of IDRs, there is a time interval between the exposure to the drug and the development of the IDILI. Usually, this takes about 1-3 months, but sometime the delay may be significantly longer. For certain drugs, the time between initial exposure and the development of IDILI is than one year (Lawrenson et al., 2000). In some cases, there is rapid development of symptoms when the drug is re-administered but this is not universal. In a few cases, IDILI is associated with fever, rash, eosinophilia. Such findings provide the clinical

evidence that the reactions may involve the adaptive immune system (Zimmerman, 1999). Moreover, antidrug antibodies or autoantibodies have been detected in the serum of patients with IDILI but little is known about their significance in the pathogenesis of liver injury (Liu and Kaplowitz, 2002). The histology of IDILI is similar to any other types of liver injury. The most common comparison is with viral hepatitis. Both conditions involve mild to moderate inflammation and lymphocytic and eosinophilic infiltration (Kleiner, 2009). IDILI caused by drugs such as isoniazid and ketoconazole are classified as metabolic idiosyncrasy (Zimmerman, 1999).

A study has shown that HLA association with IDILI (that is induced by structurally diverse drugs: flucloxacillin, co-amoxiclav, ximelagatran, lapatinib, lumiracoxib) reside on common HLA haplotypes (B*57:01, DRB1*15:01, DRB1*07:01, DQA1*02:01 and DRB1*15:01 respectively) which were present in populations of diverse ethnicity (Alfirevic et al., 2012).

1.4.9 Diagnostic tests

In some cases of drug hypersensitivity there may be laboratory findings that provide clues for the underlying mechanisms. These tests also provide a more solid ground for diagnosis.

In vivo diagnostic tests include skin tests and analysis of the eosinophil count and the presence of autoantibodies. In vitro, it is possible to detect drug-specific T-cells and the presence of certain cytokines secreted from drug-stimulated PBMCs (Gómez et al., 2012). Specific tests and their applicability domain are discussed in detail below.

1.4.9.1 In vivo tests

1.4.9.1.1 Serum tests

These include a variety of markers like tryptase level and histamine level. These markers are of value in diagnosing type I hypersensitivity reactions (Pichler, 2007)

1.4.9.1.2 Skin tests

These include **prick**, **intradermal** and **patch** tests (Bernstein et al., 2008). These tests give variable results depending on the type of reaction and the drug. They were shown to be of high sensitivity 70% and specificity 97-100% for benzyl penicillin type I reactions (Kranke and Aberer, 2009). On the other hand, low sensitivity and specificity was found with non-immediate β -lactam reactions in patients with cystic fibrosis (Whitaker et al., 2012). Padial *et al.* showed that only 9% of patients with non-immediate β -lactam hypersensitivity reactions had positive delayed intradermal tests (Padial et al., 2008). Although skin tests do not offer a predictive value for the β -lactam non-IgE dependent reactions, they are useful to exclude concomitant type I hypersensitivity reaction (Khan and Weiss, 2013).

1.4.9.1.3 HLA typing

Significant advances have been made in pharmacogenomics over the last decade. Most significantly, several forms of hypersensitivity are now known to be exquisitely linked to expression of a single HLA allele.

HLA phenotype is important when there is high linkage disequilibrium that yields high positive and negative predictive values. The famous example for HLA phenotype implementation is abacavir hypersensitivity and the association with HLA-B*57:01. HLA-B*57:01 testing is associated with a positive predictive value 47.9% and negative predictive value 100% (Mallal et al., 2008). Thus, identifying an HIV patient with HLA-B*57:01 leads to avoiding abacavir and prescribing a safer alternative. This has eliminated abacavir hypersensitivity.

1.4.9.1.4 Drug provocation test (DPT)

This test provides the gold standard to confirm or exclude the drug hypersensitivity as the patient is re-exposed to the suspect drug. However, its use in patients with positive skin tests is limited by ethical reasons as there is a risk that the patient

may generate a severe systemic reaction (Aberer et al., 2003). Moreover, the evaluation of the test result for management decision is still controversial (Romano et al., 2004).

1.4.9.2 In vitro tests

In vitro tests have the advantage over the in vivo tests that they are free of risk to the patient; however, the sensitivity and specificity of the assays have not been defined, which limits their use as for diagnosis.

1.4.9.2.1 Lymphocyte transformation test (LTT)

This test depends on proliferative responses of patients T-cells in response to drug. PBMCs are incubated for 6 days in a 5% CO₂ ventilated incubator at 37°C in the presence and absence of the culprit drug. Radioactive thymidine is added for the last 16 hrs. Later, radioactivity in drug-treated and control wells is counted which represents the degree of thymidine incorporation to DNA. Stimulation index (SI) is the ratio between the proliferative response of in drug-treated wells over the proliferative response of the medium control. SI \geq 2 is usually regarded as positive (Pichler and Tilch, 2004).

One research has assessed the sensitivity of LTT to be (74%). This is significantly higher than that of skin tests while the specificity to be 85% (Blanca et al., 2009). Other studies have confirmed the reliability of this test in the diagnosis of β -lactam allergy. (Nagao-Dias et al., 2009).

³H-thymidine uptake proliferation assay is a simple modification of LTT in which the tested cells are primed (memory) T-cells clones (TCCs). Therefore, the proliferative response is expected to be much faster and so the incubation is restricted to (48hrs).

More recently, modifications have been made to the LTT. Specifically, multiple readouts have been used to analyse different aspects of the T-cell response. These include pulsing APCs, MHC blocking and others.

1.4.9.2.2 Enzyme Linked Immunospot Assay (ELISPOT) and Intracellular Cytokine Staining (ICS)

Both assays are used to study different phenotypes of type IV hypersensitivity reactions (Scheibenbogen et al., 1997).

They are qualitative and quantitative tools to measure release rate of target cytokine(s) by specific T-cells in response to pharmacological doses of a specific drug.

- **ELISPOT assay**

The T-cells are incubated with autologous antigen presenting cells and the drug together in a membranous plate that is coated with an anti-cytokine antibody. The antibody binds the cytokine when it is released from the T-cells. Subsequently a second antibody is added to allow visualization of the bound antibody. Following addition of an enzyme substrate, dark coloured spots representative of a drug-reactive cell are visualized (Kalyuzhny, 2005). It has been estimated that ELISPOT can detect 1 in 100000 cells (Asai et al., 2000).

- **Intracellular Cytokine Staining (ICS)**

It is an assay that is reliant on flow cytometry. The assay allows the analysis of individual cells in mixed populations. T-cells are stimulated with antigen in the presence of protein transport inhibitor, which prevents cytokine release from the cells. Cells are then washed then anti-cytokine antibodies are added. Cells expressing the cytokine are counted using fluorescence labelled antibodies (Rive et al., 2013). The flow cytometer uses laser beams of different wavelengths to detect the fluorochromes and so by using different fluorochromes labelled with different antibodies we can detect multiple cytokines simultaneously (Freer and Rindi, 2013).

1.4.9.2.3 Flow cytometry

Flow cytometry has gained great importance over the last decade after the new advances, which added more capabilities for this technology. In addition, it enables us to quantify cell proliferation using CFSE staining methods (Boks et al., 2010). Moreover, it can sort cells and thus, we can enjoy having cells of specific criteria with high purity that can be utilized later in cloning assays.

1.4.9.2.4 T-cells priming and/or cloning assays

Over the last two decades, we have witnessed great leaps in our knowledge of T-cell physiology and its role in immunity, which enabled us to develop new techniques to prime naïve T-cells and/or make clones from memory T-cells. These techniques are of value when the primed (memory) drug-specific T-cells are low in number in patient blood or tissue when polyclonal T-cell responses are not detectable. Furthermore, T-cell cloning assays are of value when the anticipated drug reaction is so serious e.g., SJS and therefore any trial to induce such reaction in vivo would be unethical.

In addition, drug specific clones provide us with valuable chance to study the phenotype and function of T-cells under controlled fixed conditions and thus avoiding any bias that may ensue in vivo due to interaction of the patient with the environment.

1.4.9.2.5 Others

Other in vitro tests include **radioallergosorbent testing (RAST)**, **enzyme linked immunosorbent assay (ELISA)** or **fluoroenzyme immunoassay (FEIA)** and **basophil activation test**. These tests are all designed to measure the level of the IgE expressed in response to an allergen. Hence, they are of no value in diagnosing non-immediate drug hypersensitivity reactions (Rive et al., 2013).

1.4.10 Diagnostic approach

Type B drug reactions are a cause of significant morbidity and mortality; moreover, they are unpredictable. Type I and type IV reactions are the most common in clinical practice. Careful clinical history and phenotyping are the main pillars for diagnosis and patients' stratification and are of value to direct the choice of diagnostic tests with the outcome of identifying patients with true immunologically mediated drug reactions.

The gold standard for evaluating type I reactions involves in vivo testing such as prick and intradermal tests with validated reagents and drug provocation test.

Type IV delayed reactions are T-cell mediated involving a variety of cell types and multiple cytokines in addition to variable presentation both in type and magnitude. In vivo tests such as patch testing and intradermal testing are conducted with delayed readings together with ex vivo testing such as LTT and ELISPOT to support the diagnosis of immunologically mediated drug reactions to a specific drug. However, the sensitivity of these tests are less than 100% and so given the severity of these adverse drug reactions the patients are advised to avoid using the suspect drug and to replace it with a safer prescribed one (Rive et al., 2013).

1.4.11 The outcome of screening and diagnostic testing for immediate and delayed drug hypersensitivity reactions

Combination of the tests mentioned above are useful clinical tools for the delineation of the nature of the drug reaction, clarifying or removing the label of immediate drug reaction and providing the patient with specific valuable information about which drugs or classes of drugs can be safely taken in future. Clinical studies have highlighted that most patients labelled with immediate drug hypersensitivity can safely take the culprit drug at some time after. The most common reason is that the initial presentation was not an IgE mediated reaction; moreover, the reaction is not permanent and there is an attrition with time. Approximately, 10% of patients annually lose skin test reactivity to penicillin and

80% of patients over 5 years lose skin reactivity to amoxicillin. So, false labelling of patients as drug allergic imply that these patients may need to take more toxic and more expensive drugs which are actually unnecessary (Rive et al., 2013).

1.5 Overview on the immune system

1.5.1 Immunology

Immunology is the study of the body's defence against infection. Its origin is attributed to Edward Jenner who observed in the 18th century that the mild disease of cowpox or vaccinia could confer a protection from the fatal version of the disease. Although Jenner introduced vaccination, he knew nothing about the causative agent. It was not until late the 19th century when Robert Koch demonstrated that the disease is caused by specific microorganisms (Murphy, 2012)

1.5.2 The immune response

The immune response can be divided into two types: innate and the adaptive immune responses:

1.5.2.1 Innate immune response

The innate response is characterized by being immediate, non-specific and incapable of developing an immunological memory that can identify the pathogenic microorganisms when they re-attack the body.

Components of the innate immunity

- **Physical barriers**

These include the skin, mucous membranes, epidermis and dermis. In addition to being a physical barrier ,the skin provides low pH due to the presence of lactic and fatty acids, while the dermis produces sebum with a low ph. (Khan, 2008).

- **Chemical barriers**

Chemical barriers include endogenous chemicals like the hydrolytic enzymes within saliva and the low pH of the stomach and the vagina. One of the main elements in the innate immune response is the complement system which plays a tremendous role in combatting infections through several mechanisms which include: opsonisation, chemotaxis, cell lysis and clumping of antigen bearing agents (Parham, 2009). In addition to that there are other chemicals that mediate immunological action like the cryptidins, α -defensins, β -defensins, interferons (IFNs), and surfactant proteins A and D (Khan, 2008).

- **Cellular barriers**

Several cell groups are responsible for combatting the invading microorganisms. These are the natural killer cells, macrophages, monocytes, granulocytes (which include neutrophils, eosinophils and basophils) and dendritic cells (Murphy, 2012; Khan, 2008). These cells engulf the offending agent by the mechanisms of pinocytosis, receptor mediated endocytosis, and phagocytosis. While endocytosis is not cell specific and is carried out presumably by all cells, phagocytosis is more cell-specific and results in the ingestion of the particulate as well as the whole microorganism. The cells that are involved in the phagocytosis are the macrophages, monocytes, neutrophils and dendritic cells (Khan, 2008).

- **Innate mechanisms**

The recognition of the microbes by innate mechanisms is dependent upon common microbial patterns, which trigger the activation of innate immune cells. These include: lipopolysaccharides (LPS), mannose, fucose, teichoic acid and *N*-formyl peptides. These common microbial patterns are called pathogen associated molecular patterns (PAMPs) (Khan, 2008). These PAMPs are recognized by Toll like receptors (TLRs) that are expressed by the cells of the innate immune response in addition to other types of cells like the fibroblasts or the endothelial cells (Alegre et al., 2008). This mechanism enables the innate

immune response cells to react effectively against a wide spectrum of invading pathogens ranging from viruses to parasites (Lemaitre et al., 1996).

Upon the recognition of the pathogen by the TLRs, normally by cells residing in the proximity of the infection site, the cells start to secrete chemokines (which are defined as soluble proteins that act as a chemotactic factor for immune cells). An example includes CCL5 (Bachmann et al., 2006). Moreover, the activated resident cells and macrophages secrete soluble mediators known as cytokines such as the tumour necrosis factor (TNF- α) and the interleukins. These molecules can affect the behaviour of the cells and boost the phagocytic ability of cells of the innate immune system. As a result of the high levels of the cytokines and the chemokines, cells and plasma proteins accumulate at the site of infection causing the classical signs of inflammation which are swelling, redness, pain and heat (Moser and Leo, 2010).

1.5.2.2 Adaptive immune response

Although it is slower in onset in comparison to the innate immune response, the adaptive response is more efficient. In addition to that, the adaptive immune response is characterized by its unique feature of generating immunological memory, which entails that the second exposure of the body to the offending agent would trigger a stronger and quicker specific response in comparison to the 1st exposure (Murphy, 2012). The adaptive immune response is mediated by humoral and cellular mechanisms that are designed specifically to eliminate the pathogen (Khan, 2008). For a comparison between innate and adaptive immunity (**Table 1.5**).

Table 1.5: Comparison between the innate and adaptive immunity.		
	Innate	Adaptive
Stimulation	Not required (ready for immediate action)	Required (needs a previous exposure)
Specificity	Minimal	Highly specific Self vs non-self-discrimination Receptor mediated
Response	Within minutes No change in quality and quantity over time	Develops over days Improved by previous exposure
Memory	No	Yes
Soluble factors	Lysozyme, complement, acute phase proteins, interferons, cytokines	Antibodies, cytokines, interferons
Cells	Phagocytic leukocytes, natural killer cells	T-cells, B-cells

- **Humeral mechanisms (antibodies)**

Antibodies (also called immunoglobulins) are glycoproteins produced by activated B-cells during the immune response. They are synthesized in response to specific antigens and they can bind only the antigen which they are produced for.

Antibodies can be circulating either freely or in a bound form attached to cells by the Fc receptors (Gordon Betts J, 2016). The antibody molecule (monomer) is a Y-shaped structure that is composed of two identical light (L) and heavy (H) chains that are connected by disulfide bonds. Both light and heavy chains are composed of constant and variable regions. Papain splits the immunoglobulin molecule into three fragments of equal size. Two are the antigen binding fragments (Fab) and the third fragment is the crystallisable fragment (Fc). Antibodies are highly diverse molecules due to high variability in the Fab fragment. This variability enables each antibody to bind to a specific antigen. There are five different classes of heavy chain. The constant region of the heavy chain is identical in all antibodies of the same class. The antibodies are different according to the heavy chain of the Fc region and this difference is responsible for the functional variability of the different classes of antibodies (Khan, 2008).

Table 1.6: The 5 classes of antibodies. Adapted from (Gordon Betts J, 2016).					
	IgM	IgG	IgA	IgE	IgD
Heavy chain	μ	γ	α	ϵ	σ
Number of antigen binding sites	10	2	4	2	2
Percentage of total antibody in serum	6%	80%	13%	0.002%	1%
Crosses placenta	no	yes	no	no	No
Fixes complement	yes	yes	no	no	No
Fc binds to		phagocytes		Mast cells and basophils	
Function	Main antibody for primary response and fixes complement Monomer form is a receptor for the B-cells	Main antibody for secondary response, opsonisation, neutralises toxin	Secreted into mucous tissues, saliva and colostrum	For allergy and antiparasitic effect	B-cell receptor

Antibodies have two main roles. The first is to bind to the its specific antigen such as the virus or bacteria rendering it non-pathogenic. The second is that it triggers the recruitment of other cells or molecules to destroy the antigen bound to the antibody (Moser and Leo, 2010). See **Table 1.6**.

- **Cellular mechanisms**

The main cells involved in the adaptive immune response are B and T lymphocytes. Their classification and function is discussed below.

1.5.3 The immune system cells

The immune system is composed of various cell types with different roles. The phagocytes are involved in eliminating pathogens in both the innate and the

adaptive immune response, while the lymphoid cells confer the highest degree of specificity in recognising the self-antigens and mediating the adaptive immune response that combats the offending pathogens. All immune cells originate from the pluripotent stem cells and they develop into lymphoid cells, myeloid cells and other cells (Khan, 2008).

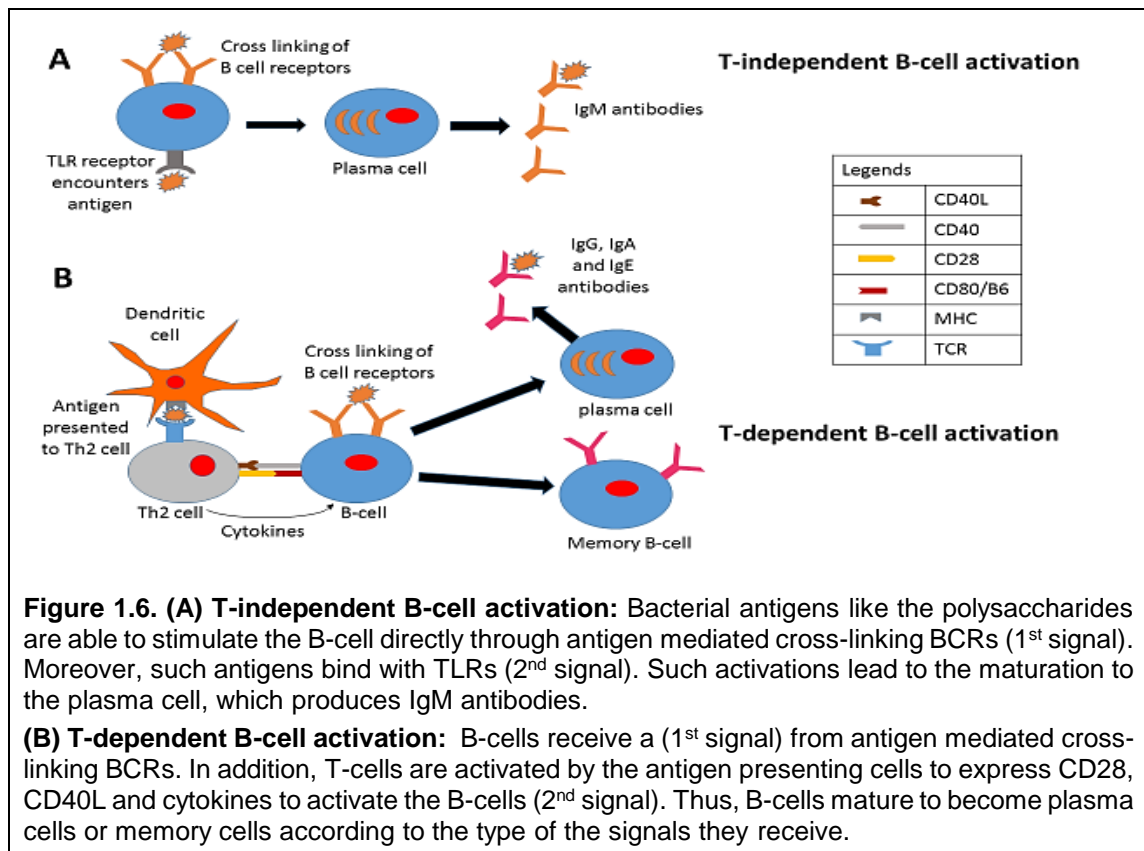
1.5.3.1 The lymphoid lineage

They constitute 20-25% of leukocytes in blood and they are divided into (Setty, 2006):

1.5.3.1.1 B-cells

They constitute 20-25% of total lymphocytes in blood (Tomasek et al., 2013). They are lymphocytes that have the unique ability to form antibodies. They develop in the bone marrow. The cell surface of the B-cell contains receptors called B-cell receptors (BCRs) that have membrane bound immunoglobulins which binds to their specific antigen. After binding of the antigen with its cognate receptor and the activation that is mediated by the helper T-cell, the B-cell transforms into plasma cell or memory cell according to the type of the signals they receive (Moser and Leo, 2010).

- **T-independent B-cell activation:** Antigens like the bacterial lipopolysaccharides activate the B-cells through cross linking of BCRs (1st signal). In addition, B-cells receive a 2nd signal from antigen binding with TLRs. B-cells activation leads to proliferation and the production of IgM antibodies mainly in a T-cell independent pattern. Such IgM antibodies have low affinity and thus display an **innate** stereotyped pattern. Overall, this type of immune response is poorly effective and emphasize the pivotal role of the T-cells in activating the B-cells to mount a potent and more specific immune response (Vos et al., 2000).



- T-dependent B-cell activation:** Antigens bind to BCRs providing the 1st signal for B-cells. The typical secondary immune response develops on multiple exposures to the same antigen and requires APCs such as the dendritic cells or macrophages that activate the Th2 cells to the specific antigen. Then the activated T-cells play an important role in activating the B-cells (2nd signal) by producing signals (both soluble and humoral) (Fazilleau et al., 2007). Because of this activation, the B-cells differentiate into plasma or memory cells. Moreover, Plasma cells start the production of large amounts of IgG, IgA and IgE antibodies in a process known as **class switching**. Such antibodies have a higher affinity with diverse functions in comparison to IgM produced by the T-cell independent activation (Allen et al., 2007). See **Table 1.6**.

1.5.3.1.2 T-cells

They constitute 75-80% of total lymphocytes in blood (Tomasek et al., 2013). T-cells are produced in the thymus; however, their precursors are found in the bone marrow. The cells that migrate to the thymus do not express CD4 or CD8+ receptors (**double negative T-cells**). Subsequently, they start to develop in the thymus and the expression of CD4 or CD8 receptors increase. At the same time, they become able to differentiate self from non-self peptides. The cells, which don't bind to the MHC/antigen complexes weakly, die by apoptosis and so the rest of the cells will undergo **positive selection** (Male et al., 2012). Simultaneously, cells that respond to the MHC class II will retain the CD4 receptor and lose the CD8 receptor and so become T helper cell. On the other hand, the cells which respond to the MHC class I receptor will retain the CD8 receptor and lose the CD4 type and so become cytotoxic T-cells. The cells then will undergo the process of **negative selection** in which the cells that bind strongly to the MHC/self-peptide will undergo apoptosis. This process is important to get rid of these cells which may mediate autoimmunity if they still present in the body. The net result is that the cells that bind weakly to the MHC/self-antigen will be allowed to leave the thymus (Rhoades and Bell, 2009). The output cells are **naïve T-cells (Th0)** which are T-cells that are considered mature but unlike the activated or memory T-cells have not encountered their specific antigen (Reviews, 2014). These cells are characterized by the expression of L-selectin (CD62L); the absence of the markers of activation CD25, CD44 or CD69; and the absence of memory CD45RO marker (De Rosa et al., 2001). They are characterized by the presence of unique receptor which is known as the T-cell receptor (TCR) which distinguishes the T-cells from other types of lymphocytes (Parham, 2009).

T-cells are divided according to the structure of the TCR molecule into two types:

1.5.3.1.2.1 TCR-1 ($\gamma\delta$ cells)

The TCR molecule is composed of γ and δ polypeptides and constitutes 5% of the T-cells in general and 10% of intraepithelial T-cells (Khan, 2008). Unlike the $\alpha\beta$ cells the $\gamma\delta$ cells are not restricted to the recognition of peptide antigens presented by the MHC molecules and their role in the immune response is less well defined than that of the $\alpha\beta$ cells (Parham, 2009).

1.5.3.1.2.2 TCR-2 ($\alpha\beta$ cells):

The TCR molecule is composed of α and β polypeptides that constitutes 95% of T-cells. They are subdivided into two groups of cells: Th (T-helper cells) that are CD4+ and the Tc (cytotoxic T-cells) that are CD8+. Th cells recognize the antigens that are presented by MHC class II, while the Tc cells recognise the antigens presented by MHC class I. Th cells can be subdivided into: **Th1 , Th2 , Th9 ,Th17, Th22 , T follicular helper cells (Tfh) and T regulatory (Tregs)** according to their cytokine secretion profile (Murphy, 2012).

- **Th1 cells:**

They secrete IL-2, IL-3, IFN- γ , TNF- α , TNF- β and GM-CSF (Khan, 2008). These cells have an important role in immune response against bacteria enclosed inside vesicles inside the macrophages. Following recognition, antigen uptake results in activation of the macrophages and an increase in their intracellular killing capacity. Th1 cells play an important role in inflammation, cytotoxicity and delayed-type hypersensitivity (Murphy, 2012).

- **Th2 cells:**

They secrete IL-4, IL-5, IL-6, IL-10, IL-13, TNF- β and GM-CSF. Moreover they support B-cell activation, isotype switching and IgE production (Khan, 2008). They are specialised in mounting immune responses at mucosal surfaces and particularly against parasitic infections. The protective mechanisms that are produced are shared with what is commonly known as “allergic responses” which

are characterized by the production of mucous, recruitment of eosinophils and the production of IgE antibodies (Murphy, 2012). While most of the Th1 cells leave the lymph nodes and travel to the site of the inflammation, Th2 cells remain in the lymph nodes (Parham, 2009).

- **Follicular helper T-cells (Tfh):**

These cells were first found in human tonsils (Breitfeld et al., 2000), in which there is a continuous exposure to antigens. Bcl6 was recognized as a master regulator for the Tfh differentiation (Johnston et al., 2009). Tfh cells are recognized by their constitutive expression of B-cell follicle homing receptor CXCR5 (Fazilleau et al., 2009). It has been shown that IL6/IL21 promotes Tfh differentiation through activation of STAT3 signalling pathway. Tfh cells are important for the development of germinal centres and the differentiation of B-cells to plasma or memory cells (Baumjohann et al., 2013).

- **Th9 cells:**

They are a group of CD4+ helper T-cells that express IL-9, IL-10, IL-21, CCL17, and CCL22. Following activation, naive CD4+ T-cells differentiate into Th9 cells in the presence of TGF-beta and IL-4. However, unlike Th2 cells, Th9 cells do not express IL-4, IL-5, or IL-13. Th9 cells play an important role in host defence against helminthic infections, yet they may have detrimental effects in the form of the development of chronic allergic inflammation, airway remodelling, and autoimmune disease (Kaplan et al., 2015), pulmonary fibrotic diseases like cystic fibrosis (Wilson and Wynn, 2009).

- **Th17 cells:**

Th17 cells play an important role in autoimmune diseases like rheumatoid arthritis, psoriasis, inflammatory bowel disease and ankylosing spondylitis (Korn et al., 2009). Th17 cells count in the peripheral blood has been found to be inversely

correlated with poor lung function in patients with cystic fibrosis (Mulcahy et al., 2015).

There are two subtypes: pathogenic Th17 (that have been induced by IL-1 β and IL-23) (Singh et al., 2013), non-pathogenic Th17 cells (induced by TGF- β and IL-6) (Lee et al., 2012).

These cells produce distinct cytokines that result in responses promoting the recruitment of neutrophils, which are effective in dealing with extracellular bacteria. These include IL-17A, IL-17F, IL-21, and IL-22 (Zambrano-Zaragoza et al., 2014).

- **Th22 Cells:**

These are a subset of CD4⁺ effector T-cell that do not express IL-17, CCL20, IL-23 R, CD161 (Th17 markers), IL-4 (Th2 marker), or IFN- γ (Th1 marker). Instead, they have a noticeable secretion of IL-22, IL-13 and TNF- α (Eyerich et al., 2009). These cells also express CCR4, CCR6, and CCR10, which allow them to reside in the skin (Duhon et al., 2009). Activated naïve CD4⁺ cells, may differentiate into Th22 cells in the presence of IL-6 and TNF- α . Extensive research have shown that these cells may be involved in the pathogenesis of psoriasis, eczema and allergic contact dermatitis (Eyerich et al., 2009).

- **Regulatory T-cells:**

These are a subset of CD4 that constitutively express CD25 and the Foxp3 transcription factor. Regulatory T-cells are involved in limiting the immune response and preventing the autoimmune diseases by blocking the activity of helper, effector and APC cells. They are divided into natural (develop in thymus) and induced Tregs (develop from naïve T-cells in periphery) (Sakaguchi et al., 2008).

They suppress the immune response by various mechanisms: They produce adenosine (which acts as a suppressive molecule), cAMP (which suppresses the

TCR-induced Ca^{2+} , NFAT, and NF- κ B signalling). They also secrete suppressive cytokines (TGF β , IL-10 and IL-35). They suppress the secretion of IL-2 and they cause cell death via perforin and granzyme-B (Schmidt et al., 2012).

- **Natural Killer T-cells (NKT cells):**

These are T-cells that express both $\alpha\beta$ TCR and markers that are expressed by the NK (natural killer) cells such as NK1.1. However, the term is used to describe a large variety of cells including NK1.1+, NK1.1–, CD4+, CD8+, CD4– and CD8– cells. They are not restricted to MHC-dependent antigen presentation, instead they recognise antigens that are glycolipids and require the CD1d. NKT-cells are a bridge between the innate and acquired immune responses. Other markers that are expressed by these cells include CD16 and CD56. Activation of natural killer T (NKT) cells results in functions similar to CD4+ and CD8+ T-cells. These cells are cytotoxic in nature and they release cytokines like IL-4, IFN- γ , GM-CSF and others. It has been found that abnormal NKT-cell function may be associated with the development of certain cancers and autoimmune disease (Khan, 2008).

- **Memory T-Cells:**

Memory cells persist after the invading pathogen has been eliminated and they have the ability to mount a quick and stronger immune response following secondary exposure of the pathogen. Memory T-cells differ from the naïve T-cells in two features: First, they recirculate through peripheral tissues rather than through the secondary lymphoid organs and thus are activated at the site of contact with the allergen by the dendritic cells and macrophages.

Second: The activation requirements are less than those of the naïve T-cells since they do not require the stringent co-stimulatory signalling that is needed to activate naïve T-cells (Parham, 2009).

When **naïve T-cells**, which are CD45RO–, encounter an antigen presented by antigen presenting cells they become activated and transform into effector cells.

Some of effector cells are long-lived memory cells expressing CD4+ or CD8+ and the characteristic receptor CD45RO. Memory cells require the presence of IL-7 and IL-15 for survival. Moreover, they require contact with self-peptides (i.e. self-MHC peptide complexes) to proliferate otherwise they undergo apoptosis. Memory T-cells subdivide into two types of memory cell, which are known as central and effector memory T-cells. Central memory T-cells express CCR7 and secrete IL-2; they remain in peripheral lymphoid tissues after restimulation. In contrast, effector memory T-cells migrate to tissues; they lack the expression of CCR7 but they express CCR3 and CCR5. Following activation, effector memory T-cells secrete large amounts of cytokines including IFN- γ , IL-4 and IL-5 (Murphy, 2012).

1.5.3.1.3 Natural killer cells (NK cells)

Natural killer cells are a group of lymphocytes identified on their ability to combat tumour cells. They develop in the bone marrow and they lack CD3 receptors expressed on T-cells and the immunoglobulin receptors on B-cells. However, they express CD56 and CD16, which is a receptor for IgG immunoglobulin. This receptor upon binding with the IgG is important in mediating **antibody-dependent cellular cytotoxicity**. They have an important role in innate immunity (Khan, 2008).

1.5.3.2 Antigen presenting cells

1.5.3.2.1 B-cells: See 1.5.3.1.1

1.5.3.2.2 Macrophages

They are large cells that differentiate from circulating monocytes after they enter tissues. In contrast to monocytes, which circulate in blood, macrophages reside in tissues. Macrophages kill the invading microorganisms by generating reactive oxygen species and through the activation of proteolytic enzymes. Moreover, macrophages secrete several cytokines that attract leukocytes to the site of

inflammation and initiate the acute inflammatory response. Macrophages also act as antigen presenting cells, presenting antigens to T-cells. Thus, macrophages are able to bridge innate and adaptive immunity (Rhoades and Bell, 2009). In addition to their role in the immune response, they play an important role in tissue repair and aging. The expression of MHC class II and co-stimulatory molecules increase dramatically in case of infection (Khan, 2008).

1.5.3.2.3 Dendritic cells

They are very important antigen presenting cells. They are distributed in small numbers in different tissues that encounter various antigenic stimuli like the skin, lining of the nose, lungs and stomach. Following activation, dendritic cells migrate to the lymph nodes to initiate acquired immune response by their interaction with B and T-cells. The dendritic cells can be divided into myeloid dendritic cells and lymphoid dendritic cells which looks like the plasma cells and so called plasmacytoid (Murphy, 2012). Myeloid dendritic cells secrete IL-12. They can be divided into two subsets, which are MDC1 and MDC2. MDC1 stimulate T-cells, while the MDC2 have a role in fighting wound infections. The lymphoid dendritic cells are similar to plasma cells and produce large amounts of IFN- α (Khan, 2008).

1.5.3.3 Granulocyte cells

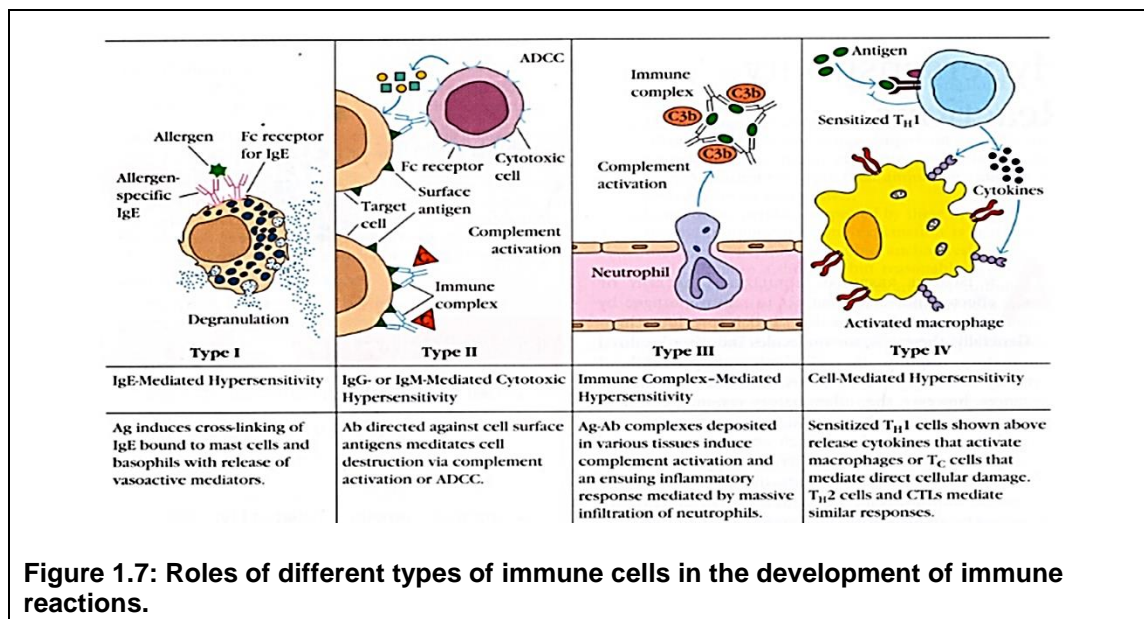
1.5.3.3.1 Neutrophils

They constitute 60-70% of blood leukocytes in healthy individuals. These cells are phagocytic by employing a number of bactericidal substances and lytic enzymes to kill the microbes. They have granules, which contain substances like myeloperoxidase, defensins, cathepsin-G and bactericidal/permeability-increasing protein, lactoferrin, cathelicidin and gelatinase. Neutrophils can regulate the function of monocytes and other lymphoid cells via the release of various cytokines including IL-1 β , IL-1ra, IL-8, TGF- β , and TNF- α . The production

of cytokines by the neutrophils is highly variable and dependent on the nature of the stimulus (Khan, 2008).

1.5.3.3.2 Eosinophils

They comprise 2% of the blood leukocytes in non-atopic individuals. They play a pivotal role in immunity to helminthic infections. Eosinophils are also capable of phagocytosis and killing ingested microorganisms but this is not their main function. Their immune function is mediated via production of reactive oxygen species (De Paulet et al., 2012), leukotrienes, prostaglandins, elastase, a plethora of cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF- α). They are present in thymus, spleen, lymph nodes, uterus and lower GI tract, but they are not present in the skin, esophagus or lungs. Eosinophils are attracted by mediators released by T-cells, mast cells and basophils. Eosinophils secrete histaminase and aryl sulfatase, which inactivates histamine and slow reacting substance of anaphylaxis (SRS-A). This will result in anti-inflammatory effects and inhibition of granulocyte migration to the site of inflammation (Khan, 2008).



1.5.3.3.3 Basophils and mast cells

Basophils are found in very small numbers in the circulation (0.01–0.3% of leukocytes). They develop in the bone marrow. Mast cells are similar to the basophils but they develop from different precursor cells in the bone marrow (Khan, 2008). Both the mast cells and the basophils release heparin. The mast cells and the basophils also release histamine and serotonin, bradykinin and the slow reacting substance of anaphylaxis. The basophils and the mast cells play a great role in mediating type I hypersensitivity reaction due to the IgE antibody that is attached to the cell membrane of these cells. So when a specific antigen binds to IgE antibody this will cause the release of large quantities of mediators (**Figure 1.7**) which causes local vascular and tissue reactions that are translated into various allergic manifestations (Hall, 2010).

1.5.4 Antigen presentation:

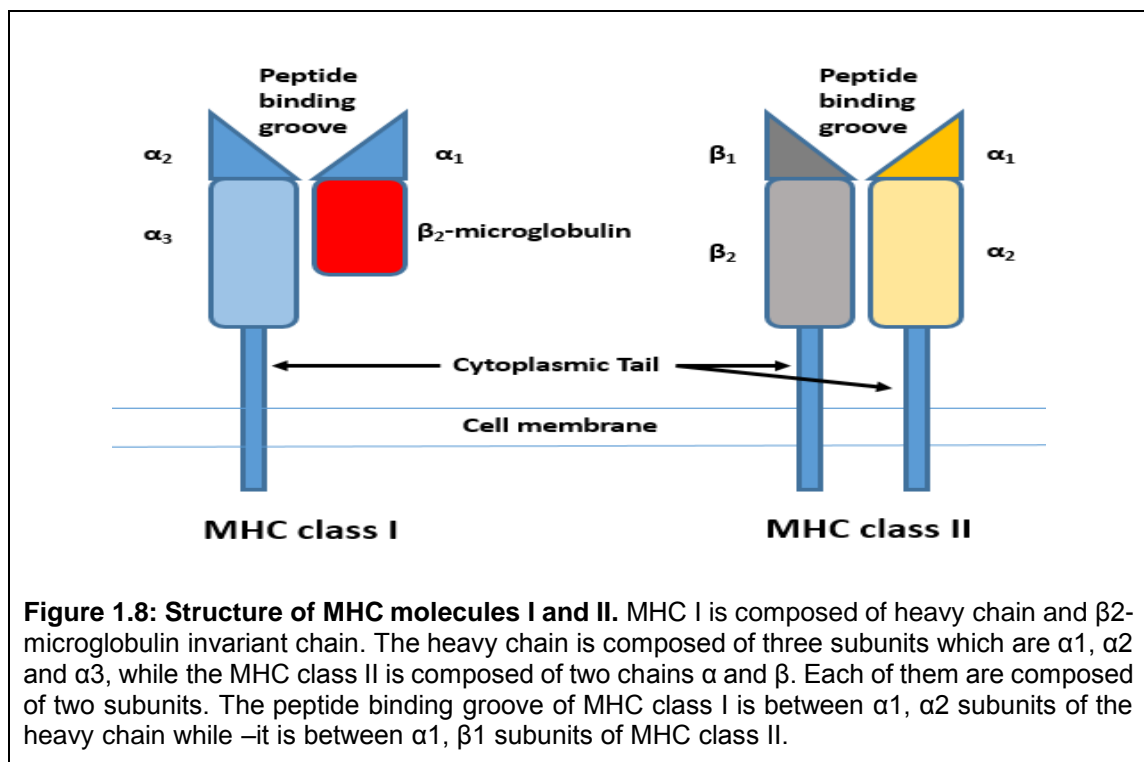
1.5.4.1 MHC structure

- **MHC class I molecule** is a transmembrane glycoprotein with a molecular weight of 43kDa. For antigen presentation it must be expressed on the cell membrane of antigen presenting cells (Coico and Sunshine, 2015). See **Figure 1.8**. It is associated with small invariant polypeptide called β_2 -microglobulin (β_2m) that is encoded by chromosome 15 (Faber et al., 1976). MHC class I molecules are composed of three extracellular domains: α_1 , α_2 and α_3 . The α_1 pairs with α_2 on the exterior of MHC I molecule while the α_3 pairs with β_2m closer to cell membrane (Coico and Sunshine, 2015).
- **MHC class II molecule** is a transmembrane glycoprotein that is composed of two chains; α and β with a molecular weight of 35 and 28 kDa respectively. See **Figure 1.8**. The structure (like MHC class I) has four extracellular components which entails α_1 pairing with β_1 on the exterior

while α_2 pairs with β_2 on the interior. Both α and β chains have cytoplasmic tails (Coico and Sunshine, 2015).

1.5.4.2 MHC class I antigen presentation

MHC class I molecules present protein fragments of intracellular origin e.g. viral proteins. Antigens are degraded into small peptide sequences by cytosolic and nuclear proteasomes. Then, by the action of the transporter associated with the



antigen presentation (TAP) these peptides are translocated to the endoplasmic reticulum (ER). See **Figure 1.9**. In the ER, the MHC class I heterodimer is composed from heavy and light chains and the peptide is the third component that is needed for stability. MHC class I complexes are transported from the ER to the cell surface for presentation to the T-cell receptor of the CD8+ (cytotoxic) T-cells (Neefjes et al., 2011). MHC I molecules that fail to associate with the peptides in the ER are returned to the cytosol for degradation (Hughes et al., 1997).

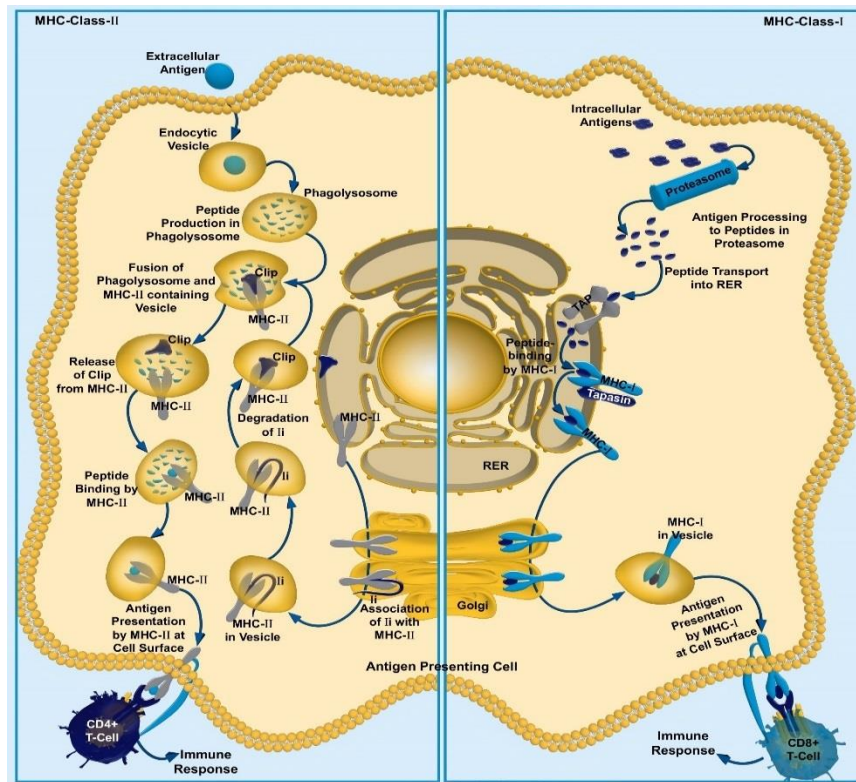


Figure 1.9: Antigen presentation by MHC I (right). Intracellular antigens are degraded by the aid of cytosolic and nuclear proteasomes into small peptides. Then, by the action of the transporter associated with the antigen presentation (TAP) these peptides are translocated to the endoplasmic reticulum (ER). In the ER, the MHC class I heterodimer will combine with the peptide. MHC class I complexes leave the ER to the cell surface to be presented to the T-cell receptor TCR of the CD8+ (cytotoxic T-cells).

Antigen presentation by MHC II (left): The transmembrane α and β chains of the MHC class II molecules are assembled with the invariant chain (li) in the ER. Then, the resulting complex is transported to an endosome where it would be digested leaving residual class II-associated li peptide (CLIP) in the peptide-binding groove of the MHC class II heterodimer. Consequently, HLA-DM facilitates the exchange of the CLIP fragment for a specific peptide derived from a protein degraded in the endosome. Finally, the MHC class II molecules are transported to the cell surface to present their antigen to the TCR of the CD4+ (helper) T cells. *Image provided courtesy of Abcam Inc. Image copyright©2015 Abcam.*

1.5.4.3 MHC class II antigen presentation

MHC class II molecules are concerned with the presentation of exogenous antigens to CD4⁺ T-cells. Previous research has shown that the transmembrane α and β chains of the MHC class II molecules are assembled with the invariant chain (Ii) in the ER. See **Figure 1.9**. Then, the resulting complex is transported to the endosomal compartment. Here the complex is digested leaving residual class II-associated Ii peptide (CLIP) in the peptide-binding groove of the MHC class II heterodimer. Consequently, HLA-DM facilitates the exchange of the CLIP fragment for a specific peptide derived from a protein degraded in the endosome. Finally, the MHC class II molecules are transported to the cell surface to present their antigen to the T-cell receptor expressed on CD4⁺ (helper) T-cells (Neefjes et al., 2011).

1.5.4.4 Cross presentation

Cross presentation acts like an intersecting mechanism between the classical two mechanisms mentioned above. It involves the presentation of exogenous antigens by the MHC class I molecules. Cross presentation is an essential mechanism for triggering the immune response against viruses that do not infect the antigen presenting cells in addition to tumours (Rock, 1996).

1.5.5 The role of T-cell receptor (TCR) in mediating immune response

1.5.5.1 TCR structure and diversity

The TCR molecules found on the surface of T-cells have an important role in recognition of antigens and T-cells activation. The pairs of α/β or γ/δ develop from the somatic DNA recombinations of germline gene segments during T-cell development.

The human T-cell α and δ chains genes loci are on chromosome 14 while the β chain and the γ chains genes loci are on chromosome 7 (Boehm and Rabbitts, 1989).

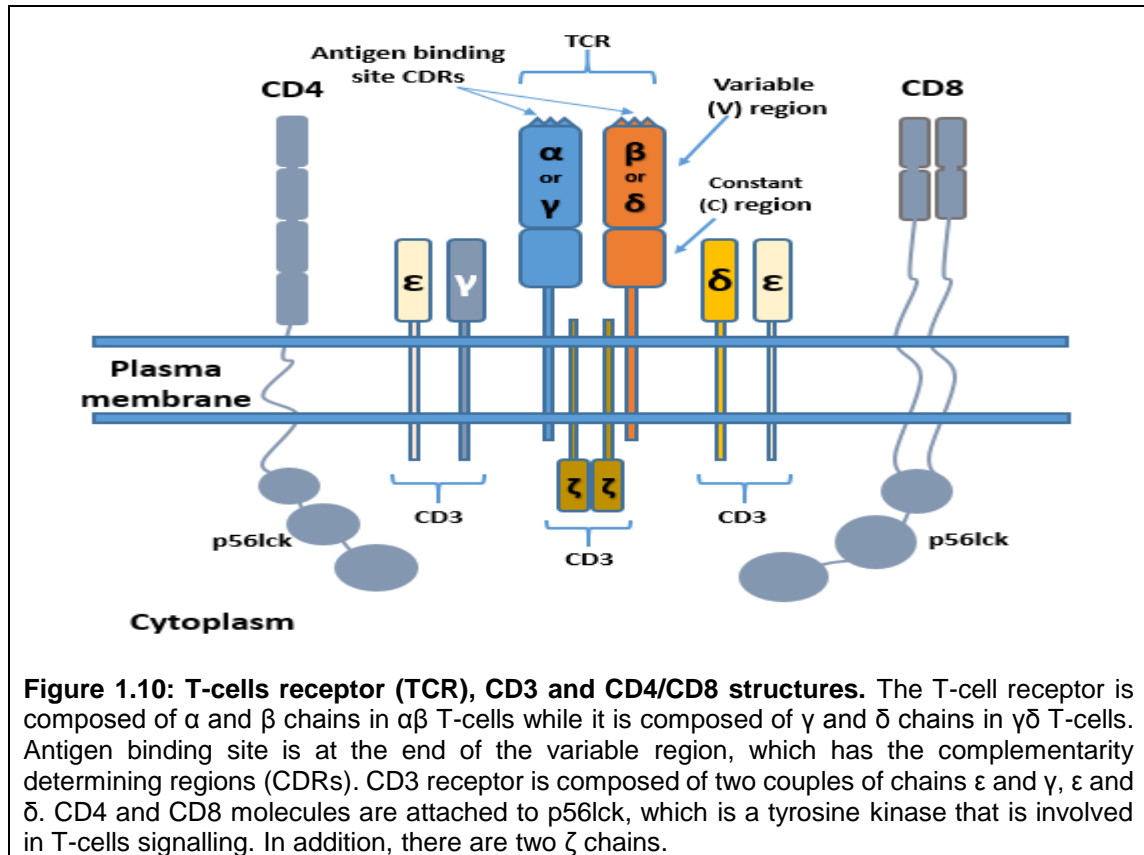
The available pool for the $\alpha\beta$ TCR comprises of 42 variable (V) and 61 joining (J) segments in the α locus and 47 V, two diversity (D), and 13 J segments in the β locus (Arstila et al., 1999). Each chain is composed of two extracellular domains: Variable (V) region and a Constant (C) region, both of which belong to the Immunoglobulin superfamily (IgSF) domain. The Constant region is proximal to the cell membrane, followed by an anchoring transmembrane region and a short cytoplasmic tail, while the variable region binds to the peptide/MHC complex. See **Figure 1.10**. The variable domain of both the TCR α -chain and β -chain each have three hypervariable or complementarity determining regions (CDRs), whereas the variable region of the β -chain has an additional area of hypervariability (HV4) that does not normally contact antigen and, therefore, is not considered a CDR.

The most important CDR is CDR3 which is responsible for recognizing processed antigen. However, CDRI of the α chain interacts with the N-terminal of the antigenic peptide while the CDRI of the β chain interacts with the C-terminal of the peptide. CDR2 is thought to recognize the MHC molecule.

The presence of multiple V/D/J gene segments in germline DNA leads to enormous diversity in TCR composition. Moreover, the insertions and combinations of template independent nucleotides at $V\beta$ - $D\beta$, $D\beta$ - $J\beta$ and $V\alpha$ - $J\alpha$ junctions would add to the diversity. Consequently, most of the diversity will accumulate in the complementarity-determining region 3 (CDR3) which is encoded by the V(D)J junction and interacts with the antigenic peptide presented by the major histocompatibility complex molecule (Stauss and Xue, 2011).

$\alpha\beta$ cells constitute approximately 95% of T-cells in human body (Girardi, 2006). Moreover, diversity in CDR3 of TCR- $V\beta$ accounts for most of the total diversity of TCRs in an individual (Laydon et al., 2015); thus, $V\beta$ sequence of CDR3 is a more

suitable and reliable marker for clonotypic analysis of T-cells than the V α sequence.



1.5.5.2 The CD3 receptor complex

For the TCR to be fully functional in it must be assembled with the CD3 multi protein complex on the T-cells. The CD3 is composed of distinct chains (γ , δ , and ϵ) in mammals and the ζ -chain. These chains (γ , δ , and ϵ) have 44-81 amino acid sequences on their intracellular site that are called *immunoreceptor tyrosine-based activation motifs* (ITAMs) which are responsible for intracellular signal transduction. The ζ -chain has three ITAMs (Flaherty, 2012).

1.5.5.3 Stabilizing molecules

The binding of the TCR to MHC molecules is of low affinity and so additional other molecules are required to stabilize the interaction. Research have shown that CD4 and CD8 molecules stabilize the interaction between the TCRs and the MHC class II and I respectively. The CD4 and CD8 molecules have a cytoplasmic tail that is capable of signal transduction to the nucleus through the phosphorylation of serine residues which triggers the activation of the p56lck kinase which in turn plays an important role in the activation of T helper and T cytotoxic cells (Flaherty, 2012).

1.5.5.4 Co-stimulatory and co-inhibitory molecules

After T-cells are activated by the first signal (i.e. an antigen specific interaction of the TCR with an antigen loaded MHC molecule), they require a second signal to amplify or attenuate the response (Pacheco et al., 2005). This signal which is antigen nonspecific is mediated by the interplay of co-stimulatory and co-inhibitory molecules expressed on both the APCs and the T-cells. See **Table 1.2**. These molecules interact actively to shape the final outcome of the immune response; either an effective immune response or anergy (Bour-Jordan et al., 2011).

1.5.5.5 Cytokines

Cytokines are polypeptides secreted by leukocytes and other cells to act principally on hematopoietic cells. The modulation of receptors with specificity for individual cytokines results in modulation of immune and inflammatory responses. Cytokines are known to control the quality and magnitude of an immune response to a given pathogen. Th1 secreting cells release IL-2 and IFN- γ . In contrast, Th2 secrete interleukins such as IL-4, IL-5, IL-6, IL-10 and IL-13. Other cytokines like TNF- α and GM-CSF are secreted by both Th cell types (Ucytech.com, 2015). Chemokines are cytokines with chemotactic action. Cytokines and chemokines may mediate their function on the cells which secrete them (autocrine), or the

adjacent cells (paracrine) or in some examples on distant cells (endocrine) (Zhang and An, 2007).

1.5.5.6 TCRs in drug hypersensitivity

- **TCR signalling**

The interaction of MHC-antigen complex with the specific TCR leads to complex chain of signalling pathways mediating T-cells proliferation, differentiation and cytokine secretion. The interaction of APCs and T-cells through accessory molecules CD4 or CD8 receptors lead to the aggregation of TCRs on the T-cell membrane with the subsequent activation of T-cells (Choudhuri et al., 2005).

The process starts with the phosphorylation of tyrosine proteins by Src kinases and Lck (Nel, 2002). TCR phosphorylation by Lck creates a binding site for the action of another tyrosine kinase called ZAP-70 (zeta chain associated protein of 70 kDa) (Smith-Garvin et al., 2009). Then, ZAP-70 phosphorylates another two important proteins namely SLP76 (SH2-domain containing leukocyte protein of 76kDa) and LAT (linker for T-cells activation). Both trigger Ca^{2+} and diacylglycerol (DAG) release with the subsequent activation of T-cells (Li and Rudensky, 2016).

- **Selective TCR expression in drug hypersensitivity:**

The first notion for the preferential TCR expression was mentioned in the pioneering analytic study of Pichler on the TCR subsets of penicillin stimulated T-cells in 1997, which showed the presence of $\alpha\beta$ T-cells in the blood of drug allergic individuals. Pichler suggested that the preferential expression of TCR may occur in drug hypersensitivity reactions (Pichler et al., 1997). A study conducted using bacampicillin showed the TCR-V β profile shifted towards V β -2, V β -3, V β -5.1 and/or V β -14 when T-cells were activated with the drug antigen in vitro (Cederbrant et al., 2000). Moreover, in lamotrigine induced hypersensitivity there was a marked dominant clonal expansion of V β -5.1 T-cells. These studies suggest that the polymorphism within the TCR genes may serve as determinants

of susceptibility (Naisbitt et al., 2003b) and that the preferential expression of TCR may play an important role in hypersensitivity reactions (Ko and Chen, 2012).

- **Restricted TCR expression in carbamazepine SJS/TEN versus polyclonal expression in abacavir hypersensitivity**

Researchers used the strong HLA-B*15:02 predisposition in carbamazepine (CBZ)-SJS/TEN as a model to explore the repertoire of TCR expressed on CBZ-specific T-cells). A restricted and the common TCR expression of V β -11-ISGSY was identified as the predominant clonotype in many individuals that developed hypersensitivity. This clonotype was present in 16 out of 19 (84%) patients with SJS/TEN, but not at all in 17 drug-tolerant patients. The clonotype was present at

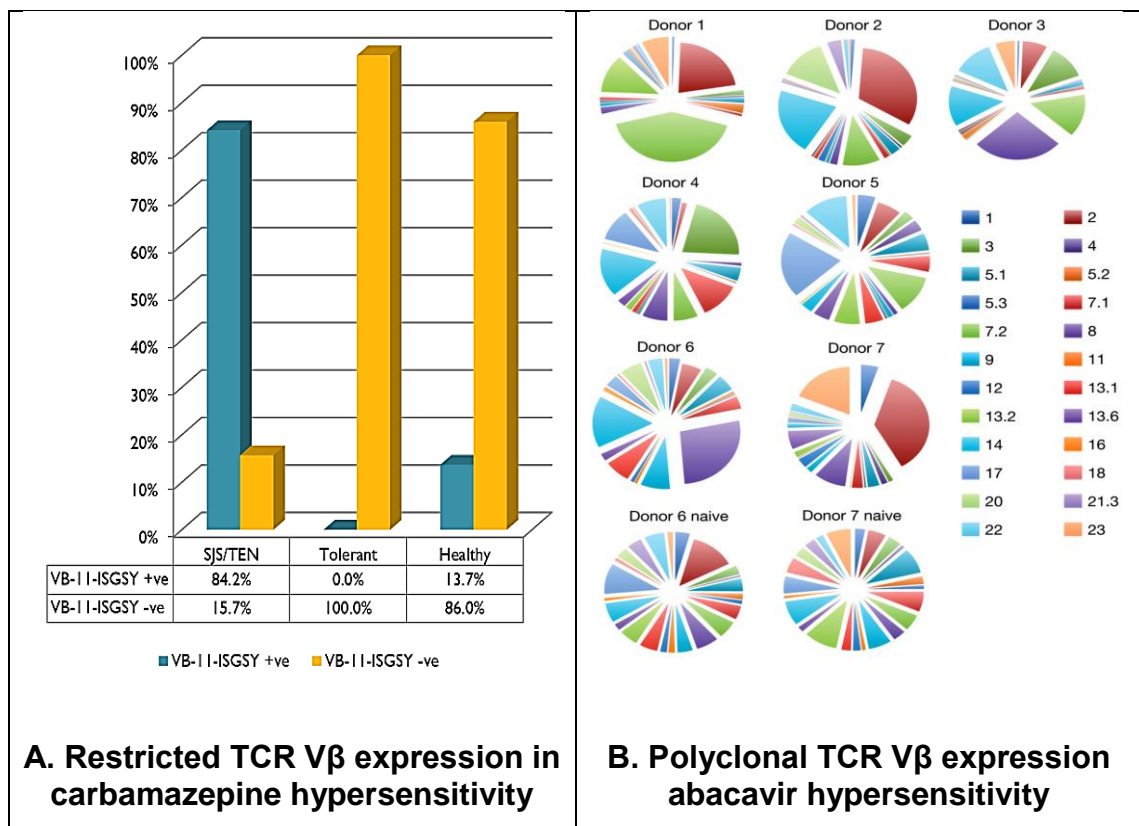


Figure 1.11: A comparison between the TCR V β expression profile of (A) carbamazepine and (B) abacavir. In the case of (A) carbamazepine the TCR-V β expression is restricted (Ko et al., 2011). While in case of (B) abacavir, it is polyclonal and there is no major TCR-V β skewing in TCR-V β expression profile from naive to primed T-cells. Reprinted by permission from Macmillan Publishers Ltd:[Nature], (Illing et al., 2012), copyright (2016).

low frequencies in healthy individuals (four out of 29; 14%) (**A**). Furthermore, T-cells obtained from the peripheral blood of healthy donors who were carriers of HLA-B*15:02 and V β -11-ISGSY showed reactivity toward CBZ

in vitro. Subsequently, the T-cell response was blocked by the addition of an anti-TCR- V β -11 antibody. Consequently, these data have highlighted the pivotal role of the TCR in the development of SJS/TEN and provided an explanation for why certain HLA-B*15:02 carriers tolerate CBZ (Ko and Chen, 2012).

On the other hand, analysis of the TCR V β expression for the abacavir-reactive CD8+ T-cells from HLA-B*57:01 positive healthy blood donors revealed a polyclonal pattern (**B**). Moreover, comparison of TCR-V β expression on naïve T-cells to that of the primed T-cells did not reveal a major skewing of the TCR-V β profile.

- **Clinical implications of drug-restricted TCR V β in comparison to the polyclonal expression**

A strong association in Han Chinese between CBZ-SJS/TEN and HLA-B*15:02 has been observed. HLA-B*15:02 was present in 98% of patients (59 of 60 patients) with CBZ-SJS, but in only 4.2% of CBZ-tolerant patients and in 8.6% of the general population (Hung et al., 2006). Similar observations were obtained from studies on patients from Hong Kong, Singapore, Thailand, Malaysia and India, where the allele is prevalent (Man et al., 2007;Mehta et al., 2009;Locharernkul et al., 2008). Moreover, in a large-scale prospective study, the use of HLA-B*15:02 screening before patients were administered with CBZ showed that the screening can effectively reduce the incidence of CBZ-SJS/TEN (Chen et al., 2011). It has been found that the HLA-B*15:02 screening has a negative predictive value of 99.9% and a relatively low-positive predictive value of 5.6% (Hung et al., 2006;Chen et al., 2011). This is due to the fact that many patients carrying high-risk alleles do not develop SJS. This observation has led to the conclusion that another factor may be implicated in the T-cell mediated

immune reactions which may contribute to CBZ-SJS/TEN development. This factor is likely to be the specific TCR clonotype that recognizes the HLA/peptide/drug complex.

Furthermore, it has been found that the clinical stage of the CBZ-SJS correlates with expression level of the dominant TCR clonotypes (CBZ-specific CDR3 sequences) in the peripheral blood mononuclear cells, suggesting that these clonotypes may be useful in evaluating the stage of the disease. Another interesting finding is that the CBZ-restricted clonotypes were undetectable in CBZ-tolerant individuals. This provides the explanation which explains why some HLA-B*1502 carriers do not develop SJS when taking CBZ (Ko et al., 2011).

The HLA-B*1502 has a low positive predictive value (5.6%) which means that many patients are unnecessarily withheld from the treatment with CBZ. Using screening with the specific TCR clonotype of the peripheral blood mononuclear cells from the HLA-B*1502-positive carriers could significantly improve the positive predictive value of identifying the high-risk individuals. Theoretically, positive predictive values of 100% could be achieved if all the specific TCR clonotypes were identified. Nevertheless, the drug specific TCR clonotypes are not present in all patients. Two of 19 with the CBZ-SJS patients did not express the specific clonotypes associated with CBZ-SJS (Ko et al., 2011). This finding would obviously lower the negative-predictive value.

Another famous example describing a role for TCR in drug hypersensitivity is the preferential expression of the TCR-V β 17 on the peripheral blood T-cells and NiSO₄ responsive T-cells isolated from inflamed tissue (Budinger et al., 2001). These data provides the evidence that T-cells carrying this particular TCR may be involved in nickel hypersensitivity (Ko and Chen, 2012).

In summary, identifying a restricted pattern of TCR-V β expression for a culprit drug can pave the way for designing new diagnostic techniques or tests to identify patients at risk of developing drug hypersensitivity reactions. Moreover, it opens

the prospect for treating drug reactions effectively through the use of specific anti-TCR-V β antibodies for the specific clonotypes of the culprit drug.

1.6 Cystic fibrosis (CF)

CF is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which is situated on chromosome 7. The CFTR gene codes for the CFTR protein that controls the chloride ion transport across epithelial cells. Research has shown that there are more than 1900 different mutations affecting this gene (Ferec and Cutting, 2012). The result of these mutations is reduced secretion of chloride and increased absorption of sodium causing thick viscous secretions (Clunes and Boucher, 2007) and blockade of secretory tubes and the affected organs such as the pancreas, salivary glands, gonads and the liver. The most significant effect is in the lung where the mucocilliary clearance of the bacteria is impaired. Patients with CF commonly present with chest infections usually *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Chinen and Shearer, 2008; Konstan et al., 2012). Moreover, the patients have difficulty in gaining weight and height (Hardin, 2004). Antibiotic treatment is regarded as a cornerstone in the therapy of the recurrent infections but their use is limited by a higher prevalence of allergic reactions which could be attributed to the high doses and long duration of use (Parmar and Nasser, 2005).

Acute chest infections are usually dealt with using intravenous antibiotics (I.V.). A β -lactam is normally used for treatment alongside either tobramycin or colistin. The most widely used drugs are piperacillin-tazobactam, aztreonam, meropenem and ceftazidime. Patients with CF have a higher volume of distribution and more rapid drug clearance. Moreover, the β -lactams fail to perfuse the bronchial secretions in sufficient concentrations (15% of blood levels) (Bergogne-Berezin, 1987). Consequently, the patients require higher doses and longer durations of antimicrobial therapy compared to standard treatments.

1.7 β -lactams hypersensitivity in patients with CF

Several studies have shown that the prevalence of β -lactam hypersensitivity is higher in patients with CF; 26-50% in patients with CF, compared with 1-10% in the general population (Koch et al., 1991; Parmar and Nasser, 2005). Hypersensitivity reactions to β -lactams are mostly non-immediate with an average delay of 9.1 days (Pleasant et al., 1994). The reactions consist of maculopapular rash, fixed drug eruptions, arthralgia, fever and/or flu-like symptoms, while Steven Johnson Syndrome and toxic epidermal necrolysis, and anaemia are extremely rare. On the other hand, immediate allergic reactions (IgE dependent) i.e. anaphylaxis and/or urticaria represent only 6% of the cases (Whitaker et al., 2012).

Piperacillin consistently has the highest rates of hypersensitivity, occurring in 30-50% of cases while other antibiotics such as mezocillin (17%), carbenicillin (7%), and imipenem (3%) have lower rates of reaction (Wills et al., 1998; Pleasant et al., 1994; McDonnell and FitzGerald, 1984). Previous research has shown that an accelerated response ensues upon re-exposure to the culprit drug, suggesting an immunological memory rather than direct toxicity. Hypersensitivity reactions to multiple β -lactam antibiotics are a common problem. Whitaker et al (2012) showed that 30% of patients had more than two previous β -lactam reactions, while 20% of patients had a single β -lactam reaction. Such patients tend to show lower lung function tests and a history of multiple and longer exposure to antibiotics (Whitaker et al., 2012).

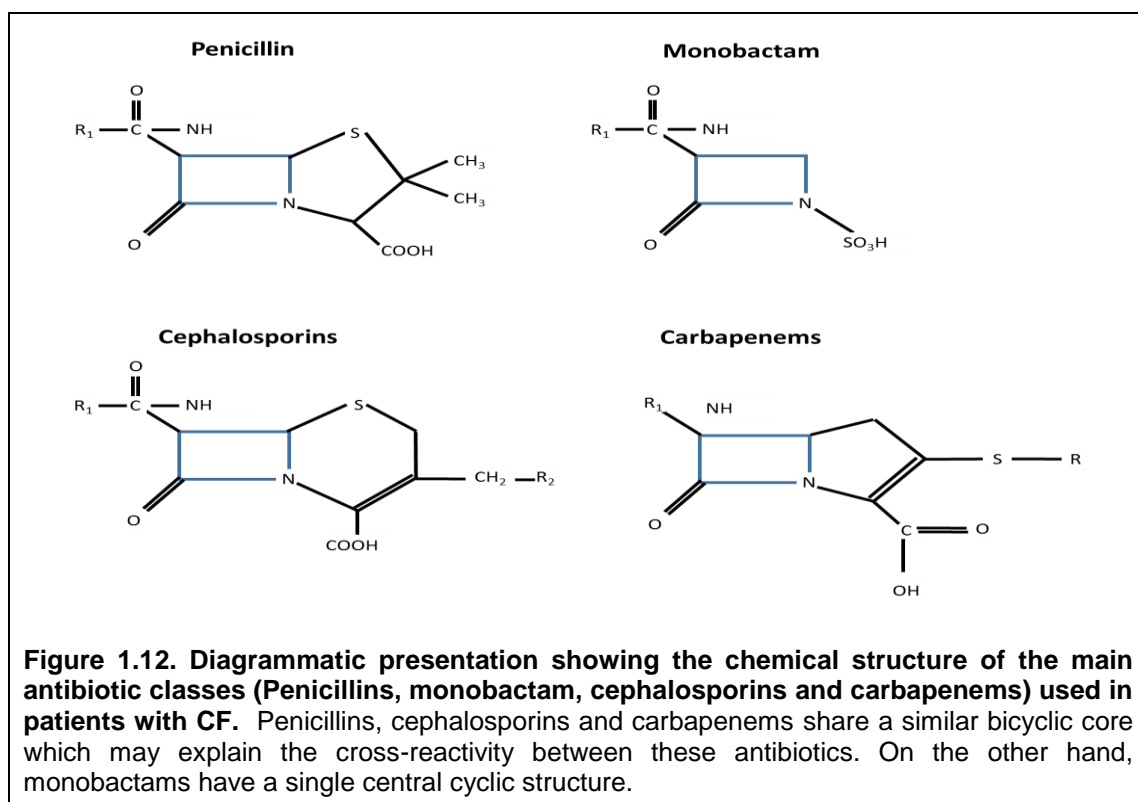
1.7.1 Cross reactions between β -lactam antibiotics

Cross reactivity between β -lactam antibiotics has been shown to be less problematic than it was thought from early studies focussing solely on similarities between structures (Ramesh and Ramesh, 2002). This picture of low cross reactivity in patients with CF is similar to that seen in patients without CF. For example, a study has shown that cross reactivity between imipenem and

meropenem in penicillin allergic patients is less than 1% (Romano et al., 2006). Aztreonam does not have the bicyclic core structure (**Figure 1.12**) of the penicillins, cephalosporins or carbapenems and therefore does not activate T-cells responsive to other drugs.

However, aztreonam has a side chain in common with ceftazidime, which signifies that a percentage of patients with ceftazidime hypersensitivity might develop a second hypersensitivity reaction when exposed to aztreonam. From a practical point of view, aztreonam is well tolerated. Only two case reports describe patients who reacted to both drugs (Moss et al., 1991; Jensen et al., 1987).

The results of several studies of cephalosporin administration in penicillin skin test positive patients have shown that there was an incidence of 4.5% of cross reactivity. The majority of reactions to cephalosporins were with drugs with similar or identical side chains (Sheppard and Welsh, 1999; Novalbos et al., 2001; Audicana et al., 1994).



1.7.2 Risk factors

A previous history of hypersensitivity reactions to penicillin accentuates the likelihood of subsequent reactions to other penicillin antibiotics by up to six fold (Ramesh and Ramesh, 2002). Despite this, there are no well-defined factors that predict the risk of developing drug hypersensitivity reactions in patients with CF. A possible hypothesis is that atopic children with CF who are skin prick positive to aeroallergens are more likely to be infected with *Pseudomonas*. Consequently, they need more frequent high doses of I.V. antibiotics that leads to a higher incidence of allergic reactions (Warner et al., 1976). Alternatively, antibiotics are commonly administered I.V. in a high dosage and for prolonged durations in a repeated schedule. Thus, increased exposure might influence susceptibility. It is possible that there is role for additional factors such as endogenous danger signals which are common in patients with CF. These include TNF- α and IL-1 which are capable of triggering dendritic cells maturation. The immune status in patients with CF is in the stage of chronic acute response due to repetitive infections; thus, it is characterized by high number of neutrophils in addition to cytokines including IL-8, IL-1B, IL-6, IL-17 and TNF- α . (Sagel et al., 2001; Bonfield et al., 1999; McAllister et al., 2005).

It is unclear whether CFTR mutations themselves mediate a pro-inflammatory state. Previous research has shown that CFTR knockout mice develop exaggerated cytokine responses after challenge with bacteria (Heeckeren et al., 1997). CFTR deficient cells have a higher tendency to express exaggerated Th2 responses when exposed to *Aspergillus fumigatus* where it is shown that these mice express higher levels of IgE, IL-4 and IL-13 when compared with controls (Mueller et al., 2011). This may explain the high prevalence of allergic bronchopulmonary aspergillosis (ABPA) in patients with CF (Skov et al., 1999). This may offer another mechanism explaining the high prevalence of drug hypersensitivity in patients with CF.

1.8 Piperacillin

Piperacillin is an extended spectrum β -lactam antibiotic of the ureidopencillin class (**Figure 1.13**). It has a wide spectrum activity against many pathogens including *Pseudomonas aeruginosa* which is one of the main life threatening infections in patients with CF (Tan and File, 1995). It is also recommended for the treatment of infections caused by most Gram +ve and Gram –ve bacteria (Perry and Markham, 1999).

Commonly piperacillin is used in combination with tazobactam (β -lactamase inhibitor) in a formulation known as Zosyn™.

1.8.1 Pharmacokinetics

Piperacillin is poorly absorbed from the gastrointestinal tract as is tazobactam and so they are both administered I.V.

Thirty percent of piperacillin binds via non-specific adsorption (non-covalent binding) to protein (Sörgel and Kinzig, 1993). The same applies for tazobactam. Protein binding of piperacillin or tazobactam is not affected by binding of the other compound. Piperacillin is widely distributed into body fluids and tissues. Mean tissue concentrations are usually 50-100% of the plasma concentration (Grayson et al., 2010).

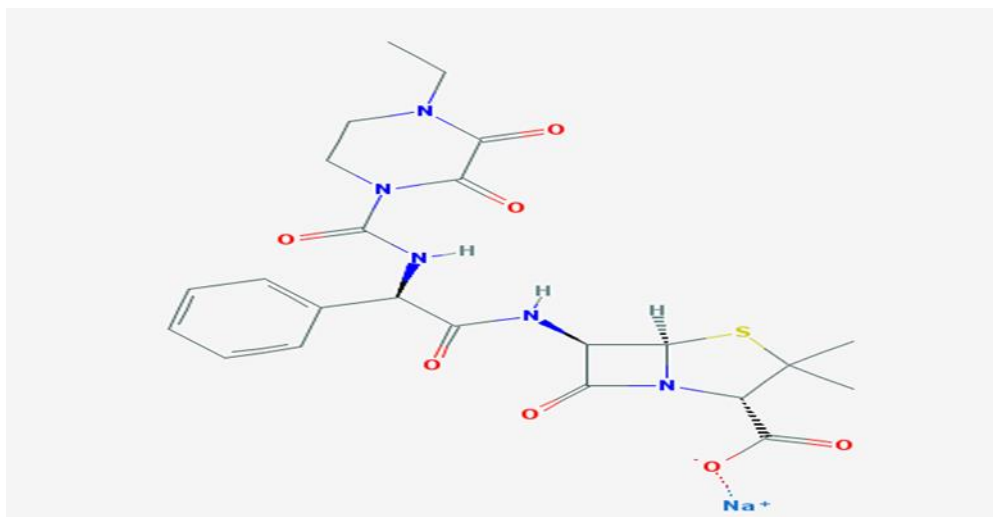


Figure 1.13: Piperacillin chemical structure.

Piperacillin is excreted mainly by renal system and to a less extent through the bile (Westphal et al., 1997). Most of the administered dose (80%) is excreted as unchanged drug while the rest is excreted as desethyl-piperacillin or desethyl piperacillin glucuronide (FDA, 2009).

Notably, the pharmacokinetics of piperacillin in patients with CF show that there is a moderate increase in volume of distribution but the clearance rate is very high which implies the need for more aggressive dosing regimens (Hayashi et al., 2010).

There is little role for metabolism in the clearance of piperacillin. Hepatic degradation of piperacillin leads to cleavage of the β -lactam ring and the formation of a minor active metabolite (*N*-desethylpiperacillin) (Wise et al., 1991).

N-desethylpiperacillin by itself is metabolized in liver to *N*-desethylpiperacillin glucuronide. Both *N*-desethylpiperacillin and its metabolite *N*-desethylpiperacillin glucuronide are detected in urine and bile (Ghibellini et al., 2007).

Piperacillin as well as its metabolite *N*-desethylpiperacillin are believed to form functional antigens capable of activating T-cells and mediating hypersensitivity reactions (Whitaker et al., 2011).

Tazobactam metabolism yields a single metabolite that does not show any pharmacological or antibacterial activities (Grayson et al., 2010).

1.8.1.1 Administration and dosage

Since it is poorly absorbed from intestine, it is administered either I.M or I.V. The usual dose is 3-4g every 4-6 hr but the maximum daily dose for adults could reach 200-300 mg/kg in divided doses daily via I.V. injection in serious infections (Hamilton, 2011).

The mean peak plasma concentration is 134, 242 and 298 µg/mL following the administration of 2, 3, and 4 g piperacillin doses respectively.

1.8.1.2 Why do we investigate mechanisms of piperacillin hypersensitivity?

Piperacillin is a β -lactam antibiotic that is frequently used to combat bacterial infections in patients with CF. However; its use is associated with a high incidence of delayed-type hypersensitivity reaction. The reactions consist of maculopapular rash, fixed drug eruptions, arthralgia, fever and or flu-like symptoms, while Steven Johnson Syndrome and toxic epidermal necrolysis, and anemia are extremely rare (Drugs.com, 2015). Thus, the problem of these reactions is not increased mortality. The main problem is the necessity to abandon the drug as a treatment option. Piperacillin has been found to be associated with the highest rates of hypersensitivity reactions in comparison to other β -lactams.

Our previous studies have identified lymphocyte proliferative responses and cytokine secretion from PBMCs isolated from approximately 75% of hypersensitive patients (Whitaker et al., 2011). In contrast, PBMCs from tolerant controls are not specifically activated (Monshi, 2013). To our knowledge, there is no evidence that tazobactam or its metabolite has any role in mediating hypersensitivity reactions in patients with CF treated with tazocin.

1.9 Hepatitis C

Hepatitis C: is a hepatic disease caused by hepatitis C virus (HCV) infection. This infection can cause both acute and chronic hepatitis infection that varies from mild illness that lasts few weeks to lifelong disease.

It is a blood borne transmitted disease mediated by transfusion of unscreened blood and blood products, careless injection practices and poor sterilization of medical equipment. Sexual transmission and transmission from mother to baby are much less common.

HCV virus infection represents a global health problem affecting 130-150 million individuals worldwide (WHO, 2015). HCV is a major cause for chronic hepatitis, cirrhosis and hepatocellular cancer. Moreover, it is the commonest indication for liver transplantation in USA (Verna and Brown, 2006).

Clinical presentation

The virus has an incubation period of 2-6 months. Approximately 80% of patients are asymptomatic (WHO, 2015). Acute symptoms usually include: fever, nausea, vomiting, dark coloured urine and faeces, jaundice, fatigue and joint pain.

Because the disease is largely asymptomatic in the acute stage, most new cases go undiagnosed until the later stages when overt liver damage has occurred (WHO, 2015).

Diagnosis

With the use of antiviral medicines, there is a high chance of cure (up to 90%) and thereby reducing the risk of death due to cirrhosis and liver cancer. However, access to diagnosis is poor (WHO, 2015).

HCV infection diagnosis is conducted usually by:

1. Anti-HCV antibody screening usually identifies people with previous infection with the virus.

2. If the result of the test is positive, a PCR test for HCV RNA is required to confirm the diagnosis.

About 15-45 % of infected people clear the virus spontaneously with their strong immune response without the need for further treatment. However, they still have HCV antibodies in their circulation (Wilkins et al., 2010).

After the patient have been diagnosed with HCV, he/she should be assessed for the degree and type of liver damage and this can be accomplished by liver biopsy and liver function test. Another important thing is that the HCV genotype should be identified since there are six genotypes and each responds to treatment differently (Al Naamani et al., 2013).

Treatment

The treatment is based mainly on:

1. Life style changes: these include measures to limit liver damage progress like stopping or lessening intake of alcohol, eating healthy balanced diet, stopping smoking and doing regular exercises.
2. Taking a combination of three medicines to stop virus replication which are a **protease inhibitor**, **pegylated interferon- α** and **ribavirin** (NHS, 2015).

The old treatment regimens were based on using **pegylated interferon- α** and **ribavirin** alone for 48 weeks which entails significant misery due to long duration of side effects of this combination which include: flu-like symptoms, depression, fatigue, anaemia, gastrointestinal symptoms and cutaneous reactions (Manns et al., 2006).

Over the last decade, there has been a great progress in the understanding of HCV lifecycle and the viral replication mechanisms that have paved the way for the development of protease inhibitor drugs (Kwong et al., 2011).

1.10 Telaprevir (VX-950)

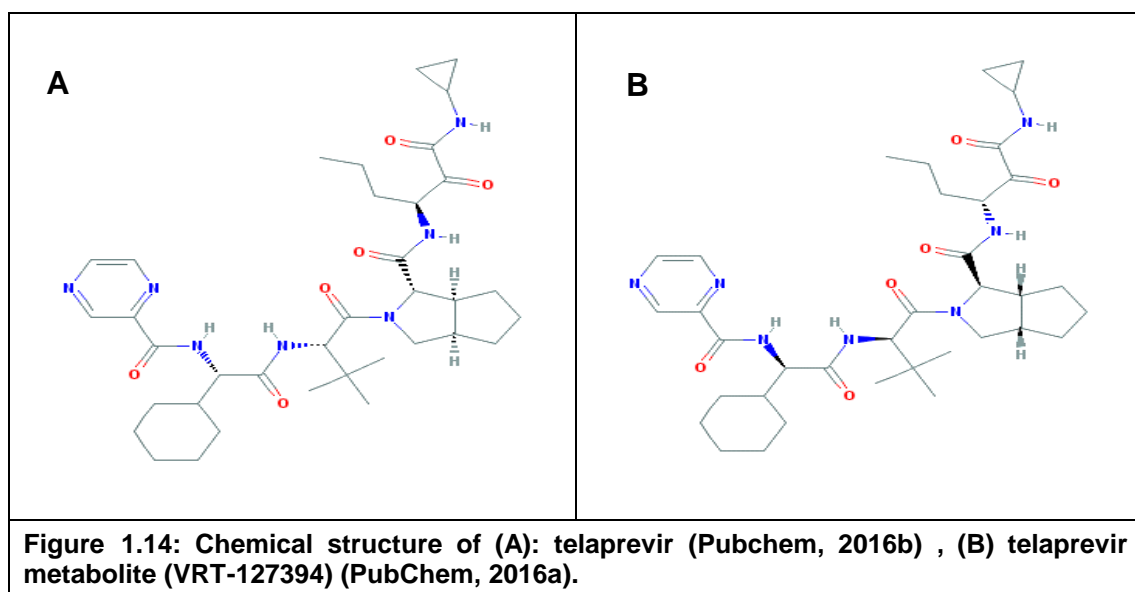
is the first anti-viral protease inhibitor that was used in the treatment of hepatitis C genotype 1. It was co-developed by Johnson & Johnson and Vertex Pharmaceuticals (Revill, 2016). It inhibits **NS3-4A serine protease** which is essential for viral replication (Jesudian et al., 2012). This enzyme is essential for the cleavage of posttranslational non-functional viral proteins into active polypeptides that are required for viral assembly (Smith et al., 2011). Another important role is that NS3-4A serine protease is vital for deactivating hepatic cellular proteins that are essential for mediating interferon cascade responsible for mounting antiviral response (Morikawa et al., 2011).

Pharmacokinetics

Absorption & bioavailability: Telaprevir is administered orally in the form of tablet or capsule with good absorption in small intestine. Maximal plasma concentration after a single dose is achieved after 4-5 hrs.

Distribution: Telaprevir is highly bound to plasma proteins (59-76% is bound). It binds mainly to alpha 1-acid glycoprotein and albumin with an inverse concentration-dependent pattern. The mean volume of distribution is approximately 252 L with 72% inter-individual variation (Vertex, 2013).

Metabolism: Telaprevir is extensively metabolized by liver through reduction, oxidation and hydrolysis, which leads to formation of multiple metabolites. The main product is VRT-127394, which is the R-stereoisomer that has 30-fold less potency than telaprevir. After administration of telaprevir, rapid conversion to VRT-127394 occurs (**Figure 1.14**) The relative ratio of telaprevir:VRT-127394 is approximately 2:1 in humans (Monitoring, 2016)



Other metabolites include VRT-0922061 (inactive isomer) and pyrazinoic acid (inactive) (FDA, 2011). Previous research has shown that CYP3A4 is the major isoform of cytochrome P450 family that is responsible for telaprevir metabolism (Vertex, 2013).

Excretion: Following administration of 750 mg single oral dose of radio-labelled telaprevir, 90% of radioactivity was recovered in faeces, urine and exhaled air after 96 hrs. Recovery of the dose was mainly in faeces (82%), with 9% in exhaled air and 1% in urine. In terms of telaprevir and its metabolite (VRT-127394), the unchanged recovered fraction was 31.9% & 18.8%, respectively (Vertex, 2013).

Recommended dose:

The recommended oral dose of telaprevir is 1125 mg (three 375 mg tabs) taken twice daily with food. Telaprevir should be administered in combination with ribavirin and interferon- α for 12 weeks (Buti et al., 2014).

Adverse effects

Dermatological

1. Very common: Rash (all grades: 56% in telaprevir combination therapy as compared to 34% in patients receiving pegylated interferon- α and ribavirin

- alone), pruritus (47% in telaprevir combination therapy as compared to 28% in patients receiving pegylated interferon- α and ribavirin alone).
2. Common: Severe rash affecting more than 50% of body surface area with eczematous component and pruritus. (4.8% in telaprevir combination therapy as compared to 0.4% in patients receiving pegylated interferon- α and ribavirin alone).
 3. Uncommon (less than 1%): Serious skin reactions (including drug reaction with eosinophilia and systemic symptoms [DRESS], Stevens-Johnson syndrome [SJS]) as compared to none who are receiving pegylated interferon- α and ribavirin. These symptoms necessitate immediate stopping of treatment (Vertex, 2013).

Telaprevir related dermatitis may occur at any time after starting treatment with a median of 15 days and interquartile range (4-41) days. The resolution of dermatological reactions is slow with a median time of 44 days and a range of (1-504) days even for patients who have discontinued telaprevir. Moreover, it has been shown that there is no correlation between the severity of the skin reactions and the plasma level of telaprevir (Roujeau et al., 2013).

These findings collectively denote that telaprevir related skin reactions may be immunological and that telaprevir is initiating an antigen-specific T-cell response that is responsible for (1) the increased frequency of reactions and (2) the appearance of severe skin reactions that are not observed in the absence of the drug.

It has been reported that 7% of patients on combination therapy discontinued treatment because of rash and/or pruritus as compared to 1% on pegylated interferon- α and ribavirin alone (McHutchison et al., 2009).

Others

Table 1.7: Clinical adverse reactions to hepatitis C regimen (pegylated interferon- α and ribavirin) in the presence and absence of telaprevir (Alkhouri and Zein, 2012).		
Side effect	Telaprevir, pegylated interferon- α and ribavirin combination, N = 1346 (%)	Placebo/ pegylated interferon- α and ribavirin, N = 764 (%)
Nausea	39	28
Anemia	36	17
Diarrhea	26	17
Vomiting	13	8
Haemorrhoids	12	3
Anal pruritus	6	1

Risk factors for telaprevir related skin reactions

Telaprevir related skin reactions have shown a significantly higher incidence in both univariate and multivariate analyses ($p \leq 0.03$) with age above 45 yrs., body mass index below 30, white race and receiving treatment for the first time. Nevertheless, there was a modest impact of these factors on incidence (the incidence difference between groups was less than 20% and often below 10%) (Roujeau et al., 2013).

Moreover, male sex was found to be a clinical risk factor i.e. it is associated with a higher incidence of more severe conditions (grade 3 dermatological reactions) (Suda et al., 2015).

Role of HLA phenotype

A study performed on 187 telaprevir treated patients measured the association between HLA phenotype with rash of any severity. Five alleles were significant at 0.05 level depending on the uncorrected p values. Two alleles (B*4402 with an odd ratio 2.43 and DQB1*0202 with an odd ratio 2.01) were found to be risk markers for severe skin reactions. However, after being corrected for multiple comparisons none proved to be significantly associated (Roujeau et al., 2013).

Role of TCR-V β

Up to date there is no scientific literature discussing any possible association between TCR-V β expression and telaprevir reactions.

Role of granulysin

Suda et al (2015) have reported an association of granulysin with the severity of telaprevir skin reactions. Moreover, there is an early rise in serum granulysin levels with the onset of severe symptoms. This increase fades within 6 days. Thus, they concluded that granulysin could be utilized as an early predictive marker for telaprevir-induced skin reactions.

Role of metabolism

Telaprevir metabolites VRT-841125 & VRT-126032 have been shown to be inducers for skin sensitization in animal studies. However, the relationship of these metabolites to the rash incidence in humans is not clear.

Pyrazinoic acid (PZA): is a major metabolite of telaprevir that was found in a small substudy phase II trial, to be at a higher plasma level in patients with severe rash as compared to the controls. However, the sample size was small with a high variation between subjects. Therefore, further work is required to explore the relationship between PZA and rash (FDA, 2011).

1.11 Aims of the thesis

The aims of the thesis are to investigate the various chemical or immunogenic factors that may play a role in mediating drug hypersensitivity reactions for piperacillin and telaprevir. This was implemented using blood samples from hypersensitive patients and healthy naïve volunteers and utilising a battery of techniques for characterization of the antigenic determinants and testing the drug specific T-cells response.

Chapter Two

**Materials
&
Methods**

2 Materials and Methods

2.1 How it works

*All chemicals and biological reagents, devices, and labware that were used in this project are listed at the appendix (**chapter 7**) with additional details about the suppliers or the manufacturers.*

Since most of my work was on piperacillin, piperacillin is mentioned in most of the following protocols as the drug under investigation. However, the same protocols can be applied on other drugs mentioned in this thesis with relevant modifications that would be discussed under related headings.

2.2 Characterisation of the response of patients T-lymphocytes to drugs

2.2.1 Cell culture medium preparation

T-cell culture medium is composed of RPMI 1640 that is supplemented with 10% human AB serum, penicillin (1000 U/ml), streptomycin (0.1 mg/ml), and transferrin (25 µg/ml). HEPES (25 mM) and L-glutamine (2 mM).

EBV-transformed B-cells (EBVs) were grown in a culture medium that is composed RPMI 1640 supplemented with 10% foetal bovine serum, HEPES (25 mM), penicillin (1000 U/ml), streptomycin (0.1 mg/ml) and L-glutamine (2 mM).

2.2.2 Isolation of peripheral blood mononuclear cells from heparinized venous blood utilising density-gradient centrifugation

Requirements:

- Venous blood from a patient.

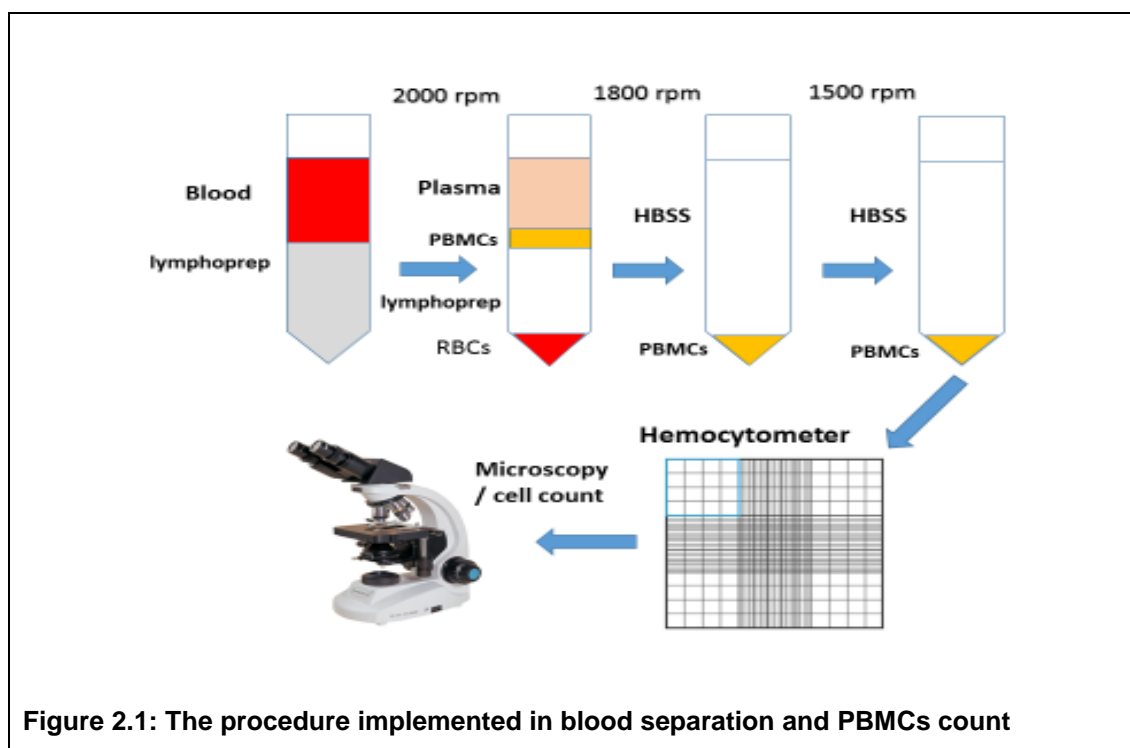
- Heparinised blood-collecting tubes.
- Blood collection set
- Lymphoprep solution
- 50 mL sterile conical tubes.
- HBSS solution
- 50mL syringe
- Kwill

Procedure:

After collecting blood by venesection, it is layered on Lymphoprep solution in a (50 mL) tube (1:1, v/v) and then spun in an Eppendorf 5810 centrifuge at 2000 rpm for 25 min with zero deceleration. After that, different layers of cells are visible. Tube shows different layers. The whitish layer of cells at the interface between the plasma and the Lymphoprep was collected, which represents the PBMCs (peripheral blood mononuclear cells) using a Pasteur pipette (**Figure 2.1**). The PBMCs were placed in a new tube and diluted 1:4 with HBSS and then spun again at 1800 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in HBSS. The tubes were spun at 1500 rpm for 10 min and the cells resuspended in a culture medium prior to counting.

PBMCs suspension (10 μ L) was added to equal volume of trypan blue (0.2 % w/v) and another (20 μ L) of HBSS was added. Then, (10 μ L) of the mixture was placed on a haemocytometer under a Leica DME microscope. Cell viability was assessed using the trypan blue, which stains the dead cells much darker than the viable cells. The percentage viability was estimated by the following equation: **percentage viability=viable cells \div total cells \times 100**. Percentage viability was $\geq 95\%$ for all the PBMCs isolations that were performed.

After cell counting, the cells are ready for culture or to be frozen using the freezing mix (cryopreservation in 80% AB serum + 20% DMSO)



2.2.3 Detection of drug-specific PBMCs responses using the lymphocyte transformation test (LTT)

Principle:

Drug specific T-cells (memory T-cells) are present in the blood of hypersensitive patients. LTT is an assay that investigates the presence of these drug specific cells. Thus, we can delineate which patients' samples are going to be enrolled in the study. Moreover, it provides valuable information about the optimal dose of the culprit drug for T-cell priming and subsequent testing (Pichler, 2014).

Proliferative responses were measured by the addition of ^3H -thymidine for the final 16h of the assay. The positive response is measured using the magnitude of the incorporation of ^3H -thymidine into DNA during cell division. The cell nucleus is then trapped on paper filter mats during cell harvesting. The mats are sealed with wax containing scintillant and then counted in a scintillation beta counter.

Requirements

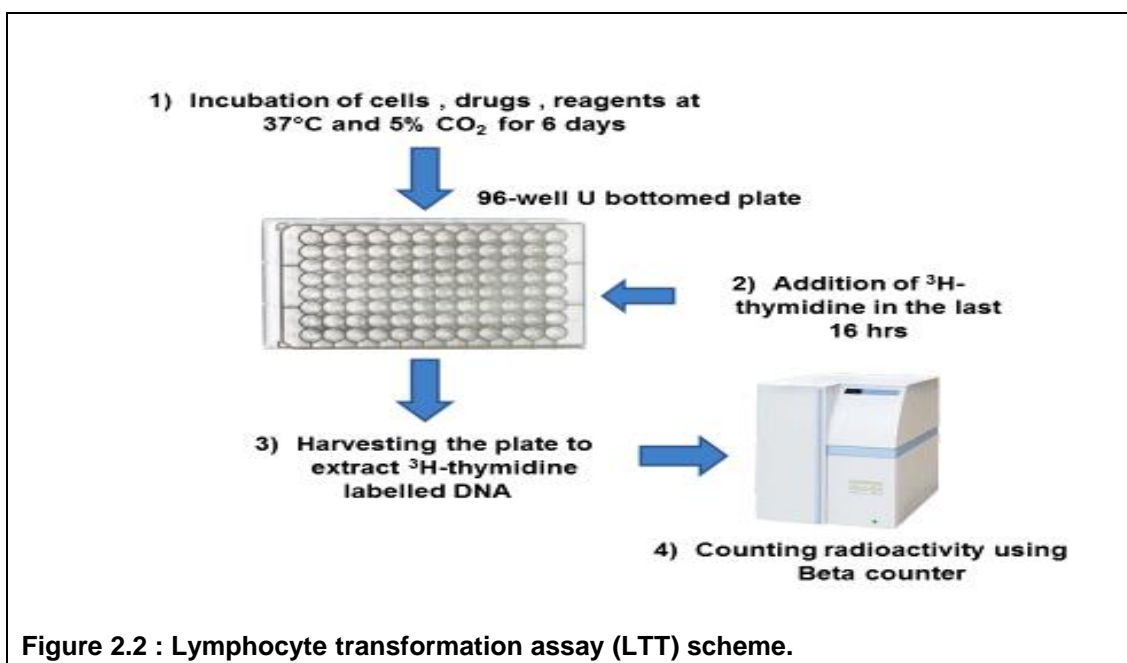
- PBMCs (0.15×10^6)/well in a 96-well U-bottom micro titre plate.
- Tetanus toxoid
- Titrated concentrations of the tested drug.
- Thymidine at concentration of 50 $\mu\text{Ci/mL}$ ($0.5 \sim \mu\text{Ci/well} = 10 \mu\text{L}$).
- Beta counter

Procedure:

Hypersensitive patients PBMCs (0.15×10^6 per well) were cultured in triplicates. The following reagents were added as three conditions:

1. Serially diluted doses of the culprit drug e.g. piperacillin ($62.5\text{--}2000 \mu\text{M}$).
2. Tetanus toxoid ($5 \mu\text{g/mL}$) as a positive control.
3. (0=medium) as a negative control.

The final volume is ($200 \mu\text{L/well}$) for all the conditions. The cultures were incubated for 6 days at 37°C and 5% CO_2 . Then, for the last 16 hrs ($0.5 \mu\text{Ci}$) of ^3H -thymidine/well was added (**Figure 2.2**). Thereafter, the cells were harvested on paper filter mats which were read by the beta counter to measure the rate of ^3H -thymidine uptake by the cells which gives a quantitative measure of proliferation in **counts per minute (cpm)**. The proliferation of the cells in piperacillin (+) was divided by the proliferation of the cells in medium (-) to calculate the **stimulation index (SI)**. An $\text{SI} \geq 2$ was accepted as a drug specific T-cells response.



2.2.4 Generation of Epstein-Barr virus transformed B-lymphoblastoid cell lines (EBVs)

Using established protocols (Neitzel, 1986; Naisbitt et al., 2003b), antigen presenting cell lines (EBVs) were generated from drug hypersensitive patients and drug naive controls as follows:

Principle:

Autologous EBVs are an essential requirement for the assays with clones T-cells to act as an antigen presenting cells. The marmoset cell line B9-58 acts as a source of the Epstein-Barr virus (EBV) which exists as circular episomal DNA inside these infected cells (5-800 copies/cell). The EBV virus selectively transforms human B-lymphocytes into an endlessly dividing, and immortalized lymphoblastoid cells i.e. EBVs (Neitzel, 1986).

Requirements

- Isolated lymphocytes total 5×10^6 cells.
- 5ml of cell-free and filtered supernatant of the marmoset cell line B9-58
- Sterile 20 mL tubes.

- 24-well sterile plates.
- Feeding medium
- Cyclosporin-A (CSA), (1 µg/mL) in DMSO

Procedure:

EBVs are generated by incubating a cell pellet of PBMCs from either patients or normal volunteers with (5 mL) filtered supernatant from the B9-58 cell line.

Cyclosporin-A (CSA) was added to the cells at (1µg/mL) to prevent T-cell proliferation and the tubes were cultured overnight (37°C, 5% CO₂). After 24hrs, the cells were spun at 1500 rpm for 5 min and the supernatant discarded. The cells were resuspended in 2ml of culture medium supplemented with (1 µg/mL) CSA. Cells were seeded by serial dilution in a 24-well plate; keeping the total volume/well at (1 mL). Cells were fed twice a week with fresh medium containing CSA (1 µg/mL). CSA addition is stopped after 3 weeks and the cells were transferred to cell culture flasks when the culture medium started to turn yellow indicating good cell growth.

2.2.5 Generation of drug-specific T-cell lines and T-cell clones from drug-hypersensitive patients

- **Requirements:**
 - Isolated lymphocytes from the involved hypersensitive patients (2mLs of cells at 1×10^6 cells/mL).
 - 96-well U-bottom micro titre plates.
 - 48-well plates.
 - T-cell growing medium.
 - PHA (1mg/mL)
 - Human r-IL-2 (100 U/mL)

2.2.5.1 Drug-specific T cell enrichment "bulk culture"

Principle:

Piperacillin hypersensitive patients blood contains primed (reactive) T-cells circulating in their blood. By incubating their PBMCs with piperacillin for 14 days, the antigen presenting cells within the PBMCs would have the chance to present piperacillin (as antigen) to T-cells. Consequently, we would expect a higher yield in terms of the primed T-cells that can be utilized in the generation of reactive specific TCCs as they will be expanded over the 14-day culture period.

Procedure:

Patient PBMCs (2×10^6 cells/well) in the 48 well plates were incubated for 2 weeks in the presence of free piperacillin (2mM) which has been shown in previous research as the optimal dose for priming and testing TCCs (Monshi, 2013). The cells were incubated at 37 °C and 5% CO₂. The cultures were fed on day 6 and day 9 with culture medium supplemented with IL-2 (60 U/mL). These cells represent the bulk cultures.

2.2.5.2 Serial dilution of bulk cultures

Principle:

The hypothesis of T cell clone generation is that each clone is generated from a single cell. To achieve this, the bulk cultures undergo serial dilution and are diluted to a degree that permits the inoculation of a single cell in each well. Proliferation is aided by the addition of irradiated, allogeneic PBMCs, IL-2 and PHA to induce the T-cells proliferation.

Procedure:

On day 14 of the incubation of the bulk cultures, the cells were harvested, washed and then counted using the trypan blue. Then the cells were serially diluted to 96-well U-bottom plates at 3 concentrations: 0.3, 1 and 3 cells/well in culture medium supplemented with IL-2 (200 U/mL), PHA (10 µg/mL) and 45 Gy irradiated,

allogeneic PBMCs (as feeder cells). PBMCs were added at a concentration of 50000 cell/ well in 100 μ L. The serial dilution cultures were incubated for 2 weeks at 37 °C and 5% CO₂ with feeding every 48 hrs with culture medium (25 μ L/well) containing IL-2 (60 U/mL).

2.2.5.3 Restimulation and the selection of well growing cells

Principle:

Incubating the T-cells with irradiated allogeneic PBMCs stimulates proliferation. This proliferative response is augmented by the presence of IL-2 and PHA. IL-2 is a cytokine that is well known as a T-cell stimulator (Liao et al., 2013). Likewise, PHA is known to induce T-cell mitosis (Movafagh et al., 2011). Using allogeneic PBMCs would provide HLA mismatch environment which adds to the strength of the stimulatory reaction in addition to their role as feeder cells.

Procedure:

The serial dilution plates were restimulated by using 45 Gy irradiated, allogeneic PBMCs at a concentration of (5×10^4 cell/mL), IL-2 (200 U/mL) and PHA (10 μ g/mL). The cultures were incubated at 37 °C and 5% CO₂ for two weeks. They were fed every 2 days with culture medium (25 μ L /well) supplemented with IL-2 (60 U/mL). Plates were assessed regularly to check for the outgrowth of TCCs. Any well containing a large cell pellet (about 2mm) was transferred into new 96-U bottomed plates and expanded into 2 wells and later into 4 wells.

2.2.5.4 Testing drug-specific T-cells proliferation using radioactive thymidine uptake assay

Principle:

It follows the same principle of LTT with some modifications to cope with a higher number of primed (memory T-cells) in the drug specific clones as compared with the hypersensitive patient-derived PBMCs. The proliferative response of (TCCs)

is stronger and faster than their precursor PBMCs; thus, the duration of the assay is limited to (48-72 hrs). Moreover, the number of cells/ well is less (5×10^5 /well) than in case of LTT and the cell incubation is conducted usually in duplicates and EBVS are used as APCs here. The interpretation of data is usually in the form of cpm without calculating SIs because clones proliferative response is highly drug sensitive and specific i.e. the proliferative response of the negative control (background) is very low as compared to PBMCs LTT.

Procedure:

The TCCs 5×10^5 /well were incubated in duplicates in the presence (+) or absence of piperacillin (-) (medium was present instead of the drug) with the addition of 60 Gy irradiated, autologous EBVs (10^4 cells/well) as an antigen presenting cells. The cultures were incubated for 48 hrs at 37°C and 5% CO₂. Then, at the last 16 hrs (0.5 µCi) of ³H-thymidine/ well was added. Thereafter, the cells were harvested on paper filter mats which were read by the beta counter to measure the rate of ³H-thymidine uptake by the cells which gives a quantitative measure of proliferation. The proliferation of the cells in piperacillin (+) was divided by the proliferation of the cells in medium (-) to calculate the **stimulation index (SI)**. An SI ≥ 2 was accepted as a drug specific cell proliferation.

2.2.5.5 Quantitative assessment of the clones reactivity to piperacillin

After identifying the drug specific clones, the response to piperacillin was assessed quantitatively. This uses the same method as above, but the T-cells were incubated with serially diluted doses of piperacillin.

2.2.5.6 Qualitative assessment of the clones reactivity to piperacillin (cross reactivity test)

Using the same protocol mentioned above, the clones that were shown to be reactive to piperacillin were tested for drug specificity. This entails incubating the lymphocytes in duplicates in the following conditions: presence (+), absence (-) of piperacillin and serially diluted doses of another drug which was normally nitroso-sulfamethoxazole (SMX-NO). Then, the SI was counted for each condition. When the SI of piperacillin was more than 2 and the SI of the other drug (in all its conditions) was less than 2, then the clone was counted as drug specific.

2.2.5.7 Restimulation and expansion of drug-specific T-cell clones

TCCs that have been shown to proliferate in response to piperacillin were further expanded in a 48 well plate in order to generate sufficient of cells to characterize their cytolytic activity, phenotype and their functionality in response to piperacillin. Expansion was performed by transfer of cells to the 48 well plates with the addition of 45 Gy irradiated, allogeneic 500000 PBMCs/well in 330 culture medium supplemented with IL-2 (200 U/mL) and PHA (10 µg/mL). Cultures were incubated at 37°C and 5% CO₂ and fed every 2 days with 330 µL/well culture medium that was supplemented with IL-2 (60 U/mL).

2.2.5.8 T-cells proliferative response with and without APC fixation

Requirements:

1. EBVs, EBVs culture medium
2. Glutaraldehyde, glycine powder
3. HBSS, filter

Principle:

According to the hapten hypothesis the drug (antigen) should bind to protein (e.g. HSA), then the formed complex (drug-HSA) would be processed and presented by the APCs (as a drug-peptide complex) to the TCRs on the T-cells via MHC molecules.

Fixing the APCs with glutaraldehyde abolishes the intracellular processing of the drug (antigen) by inhibiting protease enzyme without affecting their antigen presentation ability (Kim et al., 1985).

So, the aim of this assay is to identify the role of intracellular APC processing in mediating the T-cell response.

Procedure:

1. T-cells were suspended in HBSS in 2 million/mL concentration.
2. Glutaraldehyde (1 μ L/mL, 25%) was added to EBVs suspension.
3. The mixture is incubated at room temperature for 30s.
4. The reaction was stopped by adding glycine (0.2M) for 45s.
5. APCs were washed 3x with 25mL medium.
6. Another set of EBVs (unfixed) was prepared
7. Both fixed EBVs and unfixed EBVs are irradiated and were then used in the usual setting of the ^3H -thymidine uptake proliferation assay that is mentioned in the protocol **2.2.5.4**.

2.2.5.9 APCs pulsing assay**Principle:**

This assay explores the role of drug or protein binding with APCs in activating T-cells. In addition, it clarifies the impact of APCs drug-protein processing on T-cells activation.

The APCs are incubated with the culprit drug for variable durations 1,4,16,24 and 48 hrs then washed three times prior to incubation with T-cells. Repeated

washing will remove unbound drug and bound drug-protein adducts are the only antigen available for processing and presentation to T-cells.

When T-cells show a proliferative response upon incubation with pulsed APCs, this is indicative of a hapten mechanism. Different drugs bind to different proteins with different kinetics.

- **Time dependent peptide modification**

Note: Done only with piperacillin-HSA conjugates

Principle:

In hapten mediated drug reactions, the free (soluble) drug (in culture medium) forms drug modified peptides by binding with culture medium proteins in a time dependent manner. Thus, APCs are incubated with free drug for specified periods of time. Culture supernatant can be collected and analyzed by mass spectrometry to measure the degree of drug-modified protein formed in culture. APCs are washed several times before being irradiated to be incubated later with T-cells. T-cells proliferative response mirrors the degree of drug-protein modification.

Procedure:

1. EBVs are incubated with the culprit drug (at the same concentration that stimulates T-cells) in 24 well plate for different durations: 1,4,16,24 and 48 hrs (**Table 2.1**)
2. To measure time dependent peptide modification, supernatant samples are aspirated from the culture wells (at the end of the incubation period) to be analyzed by mass spectrometry for the degree of protein modification.

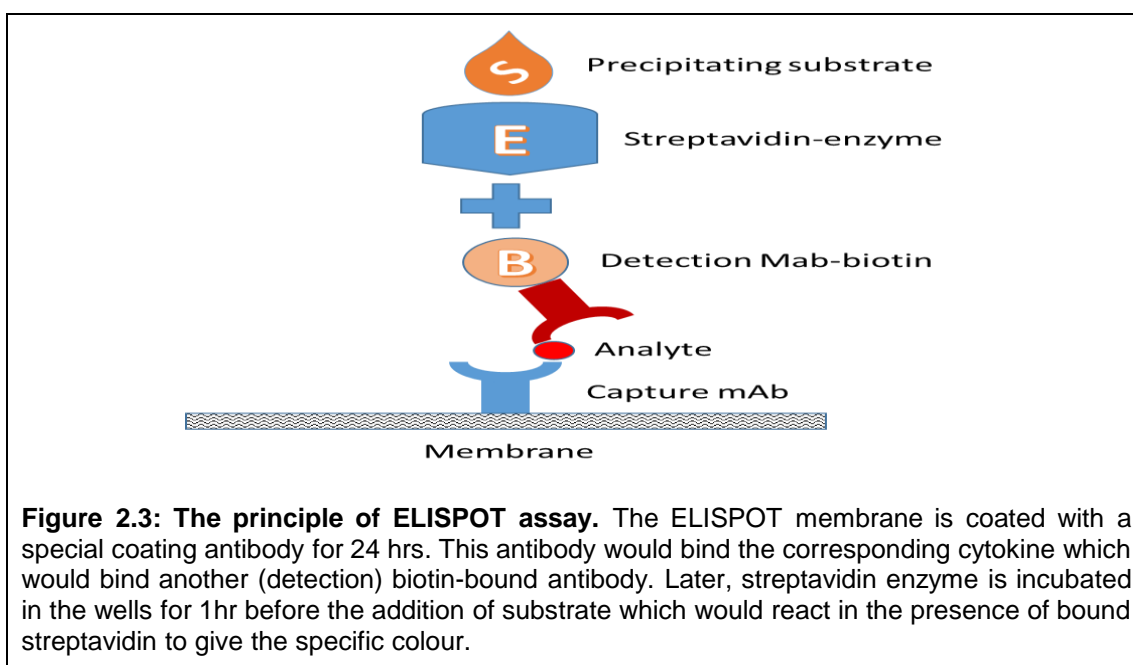
Table 2.1: The test plate design for the APCs pulsing assay.		
Conditions	Wells	
Pulsed APCs for 1 hr	50 μ L T-cells+ 50 μ L pulsed EBVs (1 hr) + 100 μ L medium	50 μ L T-cells+ 50 μ L pulsed EBVs (1 hr) + 100 μ L medium
Pulsed APCs for 4 hr	50 μ L T-cells+ 50 μ L pulsed EBVs (4 hrs) + 100 μ L medium	50 μ L T-cells+ 50 μ L pulsed EBVs (4 hrs) + 100 μ L medium
Pulsed APCs for 16 hr	50 μ L T-cells+ 50 μ L pulsed EBVs (16 hrs) + 100 μ L medium	50 μ L T-cells+ 50 μ L pulsed EBVs (16 hrs) + 100 μ L medium
Pulsed APCs for 24 hr	50 μ L T-cells+ 50 μ L pulsed EBVs (24 hrs) + 100 μ L medium	50 μ L T-cells+ 50 μ L pulsed EBVs (24 hrs) + 100 μ L medium
Pulsed APCs for 48 hr	50 μ L T-cells+ 50 μ L pulsed EBVs (48 hrs) + 100 μ L medium	50 μ L T-cells+ 50 μ L pulsed EBVs (48 hrs) + 100 μ L medium
Non pulsed APCs + medium	50 μ L T-cells+ 50 μ L non-pulsed EBVs + 100 μ L medium	50 μ L T-cells+ 50 μ L non-pulsed EBVs + 100 μ L medium
Non pulsed APCs + Culprit drug	50 μ L T-cells+ 50 μ L non-pulsed EBVs + 100 μ L culprit drug	50 μ L T-cells+ 50 μ L non-pulsed EBVs + 100 μ L culprit drug

3. The EBVs are harvested from the wells, washed twice and then irradiated with 60 Gy and then incubated at (10000 cell /well/50 μ L) in 96- well test plate.
4. Wells with non-pulsed irradiated with (60 Gy) EBVs are prepared.
5. (50000/well) T-cells, are added.
6. Culprit drug is added dissolved in medium (100 μ L).
7. Medium (100 μ L) is added to control wells.
8. The plate is incubated for 48 hrs at 37°C and 5% CO₂. For the last 16 hrs ³H-thymidine (0.5 μ Ci)/well was added.
9. The plate is harvested and read using Beta counter.

2.2.6 Quantitative and qualitative measurement of cytokine secretion by T-cell clones using ELISPOT

Principle:

The **Enzyme-Linked ImmunoSpot (ELISPOT)** assay is a very sensitive assay that measures the level of cytokine secretion at single cell level. This assay entails culturing the cells on a membrane containing plate that is coated with a specific capture antibody for the cytokine (**Figure 2.3**). When the cells secrete the specific cytokine it binds to the capture antibody. Then, after washing the plate, a biotinylated antibody is added which binds to cytokine. The binding can be visualized adding Streptavidin-enzyme conjugate (usually alkaline phosphatase) which reacts with the substrate (BCIP/NBT). Such a reaction produces a colour change in the form of spots (typically black) which can be counted visually with the aid of microscope or by the use of a spot counter. Each spot represents a single cytokine producing cell. The limit of detection can be as low as 1 cell in 100,000 (Mabtech, 2014). The ELISPOTs which were carried out were performed according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden)



2.2.6.1 ELISPOT assay for: interferon-gamma, interleukin-5, interleukin-13, perforin and granzyme-B

Requirements:

- Lymphocytes from drug hypersensitive patients or specific TCCs.
- ELISPOT well strip plate.
- Coating antibodies, detection antibodies (for IFN- γ , IL-5, IL-13, perforin and granzyme-B) are mentioned in **Table 2.2**.
- Streptavidin ALP (alkaline phosphatase).
- Detection substrate for the ELISPOT: BCIP/NBT plus.
- Ethanol 35%, HBSS, sterile dH₂O, drug of interest, PHA, PBS, FBS and SMX-NO.
- ELISPOT reader.

Procedure:

- **Coating:** on day 0:
 1. Ethanol (15 μ L, 35%) was added per well of the ELISPOT plate and left for one minute.
 2. The plate was washed 5X with (200 μ L/well) sterile dH₂O.
 3. Coating antibody (100 μ L/well) was added and incubated overnight at 4°C.
- **Cell Incubation:** On day 1:
 1. The excess antibody was washed using 5X of 200 μ L HBSS.
 2. The plate was blocked by adding culture medium (200 μ L/well) for more than 30 min.
 3. After washing the medium with 5x HBSS, 5x10⁴ /50 μ L T-lymphocytes, 60 Gy irradiated 1x10⁴ irradiated EBVs/50 μ L, medium (-), serial dilutions of piperacillin (+), PHA (5 μ g/ μ L) as a positive control and SMX-NO as an irrelevant antigen (50 μ M/mL) were added to the wells.
 4. The plate was wrapped in aluminium foil and incubated in 37 °C and 5% CO₂ for 48 hrs.

Table 2.2: Common ELISPOT cytokines with the concentrations of their reagents								
	Coating			Detection - primary Ab			Secondary Ab	Substrate
Cytokine	Name	Stock conc.	Final conc.	Name	Stock conc.	Final conc.	Name	Name
IFN- γ	1-D1K	1 mg/mL	15 μ g/mL	7-B6-1-biotin	1 mg/mL	1 μ g/mL	Streptavidin-ALP, 1:1000 dilute	BCIP/NBT
IL-5	TRFK5	1 mg/mL	15 μ g/mL	5A10-biotin	1 mg/mL	1 μ g/mL		
IL-13	IL13-I	0.5 mg/mL	10 μ g/mL	IL13-3-biotin	0.5 mg/mL	1 μ g/mL		
Perforin	Pf-80/164	1 mg/mL	30 μ g/mL	Pf-344-biotin	1 mg/mL	1 μ g/mL		
Granzyme-B	GB10	1 mg/mL	15 μ g/mL	GB11-biotin	1 mg/mL	1 μ g/mL		

- **Spot Detection:** On day 4:
 1. The plate was washed 5x with (200 μ L) of PBS.
 2. Detection antibody (100 μ L/well) was added and incubated for 2 hrs at room temperature.
 3. The plate was washed 5x with (200 μ L) PBS.
 4. Streptavidin ALP (100 μ L/well) was added and incubated 1 hr at room temperature.
 5. Finally, the plate was washed 5x with (200 μ L) PBS and thereafter (100 μ L/well) of detection substrate (BCIP/NBT) was added.
 6. After waiting until the spots emerge (which usually takes about 13-15 min) the reaction was stopped by washing the plate with tap water and then the plate was rinsed and kept in dark until it dried.
 7. When the plate is dry it was analyzed by the ELISPOT reader to count the spots.

2.2.6.2 ELISPOT for FAS Ligand (FASL)

Requirements:

- Lymphocytes from drug hypersensitive patients or specific TCCs.
- ELISPOT strip plate.

- Capture antibody, streptavidin ALP (alkaline phosphatase), detection substrate for the ELISPOT: BCIP/NBT plus.
- HBSS, sterile dH₂O, PHA, PBS, BSA, dry skimmed milk, 70% ethanol, Tween 20.
- ELISPOT reader.

Procedure:

- **Day 0 (Coating):**

1. The PVDF plate was coated with (25 μ L, 70%) ethanol for 30 second at room temperature.
2. Wells were emptied. Then, plate was washed 3x with (100 μ L/well) of HBSS.
3. Capture antibody (100 μ L) was pipetted and then added to (10 mL) of HBSS and mixed well. Then (100 μ L) of the mixture was added per well. The plate was covered and incubated overnight at +4°C.

- **Day 1 (Cells Incubation):**

1. Wells were emptied and washed once with (100 μ L) of HBSS.
2. Skimmed dry milk (100 μ L, 2%) in PBS was added per well. Then plate was covered and incubated for 2 hrs at room temperature.
3. The plate was emptied and washed once with (100 μ L /well) HBSS. Then tapped against paper towel.
4. T-cells (5×10^4 in 50 μ L medium), 60 Gy irradiated EBVs (10^4 in 50 μ L medium) and (100 μ L) of piperacillin were added per well.
5. The plate was covered in AL foil and then incubated in 37 °C and 5% CO₂ for 48 hrs.

- **Day 3 (Spot Detection):**

1. Wells were emptied by flicking the plate over the sink and tapping it over a paper towel.
2. PBS-0.1% Tween 20 (100 μ L)/well was distributed and let sit for 10 min at +4°C.

3. Wells were washed 3x using (100 μ L/well) of PBS-0.1% Tween 20.
4. The lyophilized detection antibody was reconstituted by mixing the lyophilized antibody with (0.55mL) of distilled water.
5. For one plate (100 μ L) of the reconstituted detection antibody into (10 mL) of PBS containing 1% BSA. Then (100 μ L/well) of the mixture was dispensed. The plate was covered and kept for 1 hr and 30 min at 37°C.
6. Wells were emptied and washed 3x with (100 μ L/well) PBS-0.1% Tween 20.
7. Streptavidin ALP (100 μ L/well, dilution 1/1000) in each well was dispensed. Then the plate was sealed and incubated for 1 hr at 37°C.
8. Wells were emptied and washed 3x with (100 μ L/well) PBS-0.1% Tween 20.
9. Ready-to-use BCIP/NBT (100 μ L) was dispensed in wells. Let the reaction to go for about (2-10) min at room temperature. The spots formation is monitored visually.
10. The plate was rinsed 3x with distilled water to stop the reaction. Then the plate is tapped on absorbent water. The plate was left to dry at +4°C overnight in the dark.
11. Next day the plate was read by the spot reader.

2.2.7 The quantitative and qualitative measurement of the T cell receptors and chemokine receptors using Fluorescence-Activated Cell Sorting (FACS)

Principle:

The method of flow cytometry was first described by Wolfgang Göhde in 1968, is based upon the focusing of laser beams of particular wavelengths on the cells of interest which could be stained with extracellular or intracellular fluorescent stains.

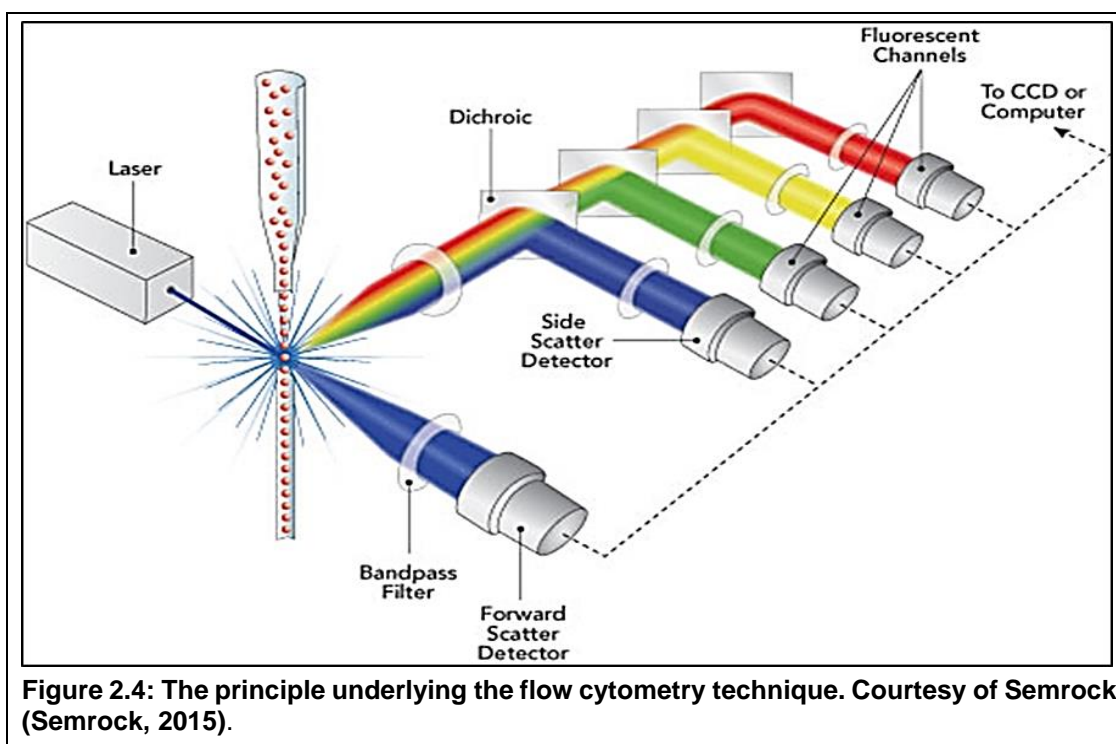


Figure 2.4: The principle underlying the flow cytometry technique. Courtesy of Semrock (Semrock, 2015).

This is followed by analyzing the cellular response to these beams in terms of distraction and fluorescence. These fluorescent stains were bound to antibodies which were specific to the receptors of the cells of interest. When they were exposed to the laser beam of certain wavelength, these stains emit light in a specific wavelength which can be detected by specific detectors which can convert the light energy to electrical signals that were measured to assess the degree of expression of such receptors (**Figure 2.4**). In some flow cytometry machines the technique is more advanced that it includes a system for sorting the cells (not used in my project) which is based on charging the cells with different electrical charges. Then, each type of cell can be pulled by an electrical field into a collecting tube depending on its charge.

Requirements:

- Non sterile FACS tubes
- FACS buffer (10% FCS, 0.02% sodium azide in HBSS)

- Fluorescence labelled antibodies for CD3, CD4, CD8, CD45RO, CD45RA, TCR-V β s and chemokine receptors.

Procedure:

The protocol mentioned below is a general protocol for all the FACS experiments that I have conducted, however, it was modified according to the requirements of each experiment in terms of the studied markers or receptors or cells. Such modifications will be discussed under the related headings.

Cells labelling:

The cells were harvested and suspended in a culture medium. Then (50ul) of the cells were transferred into non-sterile FACS tube. The fluorescent stains bound antibodies were transferred into the FACS tube in a dose that complies with the protocol issued by the manufacturer as shown in **Table 2.3**, **Table 2.4** and **Table 2.5**. Then the FACS tubes were incubated for (20 min) at 4°C (except for those of the TCR-V β which should be left at room temperature) in the dark. After the incubation, (500 μ L) of FACS buffer is added per tube. This is followed by centrifugation at 1500 rpm for (5 min). The supernatant is discarded and the cells were resuspended in (200 μ L) FACS buffer if they were to be tested by the flow cytometer on the same day. Otherwise, they should be fixed in (4%, 200 μ L) paraformaldehyde and kept in the fridge.

Compensation Preparation:

If the experiment includes multicolour staining, then compensation should be prepared. The pupose of compensation is to optimise the flow cytometry machine readings of various lasers by wiping out the interference of the fluorescent dyes signals. Compensation kit comes as 2 droppers which contain beads that can be stained with the fluorochromes antibodies used in the FACS.

FACS buffer (400 μ L) was added to one non-sterile FACS tube. Then, one drop of each compensation beads droppers were added to the tube. After shaking, (100 μ L) of the mixture was transferred to non-sterile FACS tubes and the number

of the tubes equals the number of the colours of the fluorescent antibodies used in the experiment +1 (as a control). For example, an experiment was conducted using CD3-APC, CD4-FITC and CD8-PE. Then, (3 μ L) of the fluorescent stain labelled antibody that were used in labelling the cells were transferred to each tube. i.e. (3 μ L) of CD3-APC to 1st tube, (3 μ L) CD4-FITC to the 2nd tube and (3 μ L) CD8-PE to the 3rd tube while the 4th tube was left without stain as a control. The tubes were incubated at 4°C.

When the cells or the (compensation beads) were analyzed on the flow cytometer, they appear as dots (dot plot) on the screen. Such dots need to be gated using forward scattering and side scattering into populations. Then these populations are gated again according to the fluorochrome stain which the marker of interest is attached to. In this way we can separate the cells into groups of certain criteria e.g. memory cells, CD4 or CD8 cells. The data were represented in the form of percentages (or in the case of chemokines, the cells appear as histogram and the results are expressed in terms of mean fluorescence index).

2.2.7.1 Characterisation of the phenotypic profile of T-cell clones of hypersensitive patients

The clones were characterized using the same protocol mentioned above under **heading 2.2.7** but with implementation of the scheme highlighting the doses of the reagents outlined in the **Table 2.3**. The cell clones were T-cells if they bind CD3 antibody and they were CD4 or CD8 depending on the type of the receptor antibody binding whether they bind to CD4 antibody or CD8 antibody, respectively. T-cells are naïve if they are CD45RO–ve while they are memory T-cells if they are CD45RO+ve.

Table 2.3: Protocol scheme for labelling of the T-cells to characterize their phenotypic profile.		
Tubes labelling	Compensation	Incubation
Tube 1=None	FITC, PE, APC, and PerCP-Cy5.5	20 min on ice (4°C), in the dark.
Tube 2=CD3-APC, CD4- FITC, CD8-PE (3 µL of each antibody)		
Tube 3=CD45RO-PerCP-Cy5.5, CD45RA-FITC (3 µL of each antibody).		

2.2.7.2 Characterisation of The TCR-V β of T-cell clones of hypersensitive patients

After characterising the clones phenotypically in terms of their CD3, CD4, CD8, CD45RA and CD45RO surface markers, TCCs were characterized in terms of their TCR-V β expression using the flow cytometry. The clones were labelled using the TCR-V β kit reagents mixture. Each vial contained a mixture of three fluorochrome labelled monoclonal antibodies. Using the same protocol mentioned in **heading 2.2.7**, T-cells were labelled with TCR-V β reagents as mentioned in the scheme shown in **Table 2.4**.

Table 2.4. scheme of labelling tubes for TCR-V β of TCCs (Beckman Coulter, 2015).					
Tube number	Reagent vial to be added	TCR-V β to be tested	Fluorochromes attached	Compensation used	Incubation
1	A (5 μ L)	V β 5.3	PE	FITC, PE	20 min in room temperature, in the dark
		V β 7.1	PE+FITC		
		V β 3	FITC		
2	B (5 μ L)	V β 9	PE		
		V β 17	PE+FITC		
		V β 16	FITC		
3	C (5 μ L)	V β 18	PE		
		V β 5.1	PE+FITC		
		V β 20	FITC		
4	D (5 μ L)	V β 13.1	PE		
		V β 13.6	PE+FITC		
		V β 8	FITC		
5	E (5 μ L)	V β 5.2	PE		
		V β 2	PE+FITC		
		V β 12	FITC		
6	F (5 μ L)	V β 23	PE		
		V β 1	PE+FITC		
		V β 21.3	FITC		
7	G (5 μ L)	V β 11	PE		
		V β 22	PE+FITC		
		V β 14	FITC		
8	H (5 μ L)	V β 13.2	PE		
		V β 4	PE+FITC		
		V β 7.2	FITC		

2.2.7.3 Characterization of chemokine receptors expression of T-cells clones of hypersensitive patients

Using the same protocol for labelling the T-cells mentioned under the **heading 2.2.7**, TCCs were characterized in terms of chemokine receptors expression according to the scheme shown in **Table 2.5**.

Table 2.5: The labelling of TCCs by the fluorescence labelled antibodies for various chemokines.					
Tubes	APC	FITC	PE	Compensation	Incubation type
1	None	None	None	APC, FITC, PE	20 min on ice (4°C), in the dark
2	CXCR3(3µL)	CCR5 (3µL)	CCR1 (3µL)		
3	CCR2 (3µL)	CCR3 (3µL)	CCR4 (3µL)		
4	CCR9 (3µL)	CCR8 (3µL)	CCR10 (3µL)		
5	CCR6 (3µL)	CLA (3µL)	CXCR6 (3µL)		
6	None	CD69 (3µL)	E-cad (3µL)		

2.3 Characterisation of the response of naive volunteer T-lymphocytes to drugs

In this part of the project, we tried to characterize the response of T-cells primed to piperacillin that were collected from normal (naive) volunteers. Blood was taken from three healthy volunteers (not allergic to piperacillin).

PBMCs were separated from the blood according to the protocol mentioned under the **heading 2.2.2**

Note: In this part of the study which focuses upon the responses of naive volunteer derived T-cells, I use the terms free piperacillin primed T-cells and SMX-NO primed T-cells to denote T-cells which have been subjected to the priming technique of free piperacillin and SMX-NO respectively. These cells are not

necessarily expected to be primed when analyzed by their responses by the battery of assays mentioned below (i.e. they could be primed or not) but the nomenclature is used to differentiate between them and the naïve T-cells. Eventually, the assays mentioned below will confirm their priming status in terms of specificity and the magnitude of the response.

Ds-T-cells priming assay:

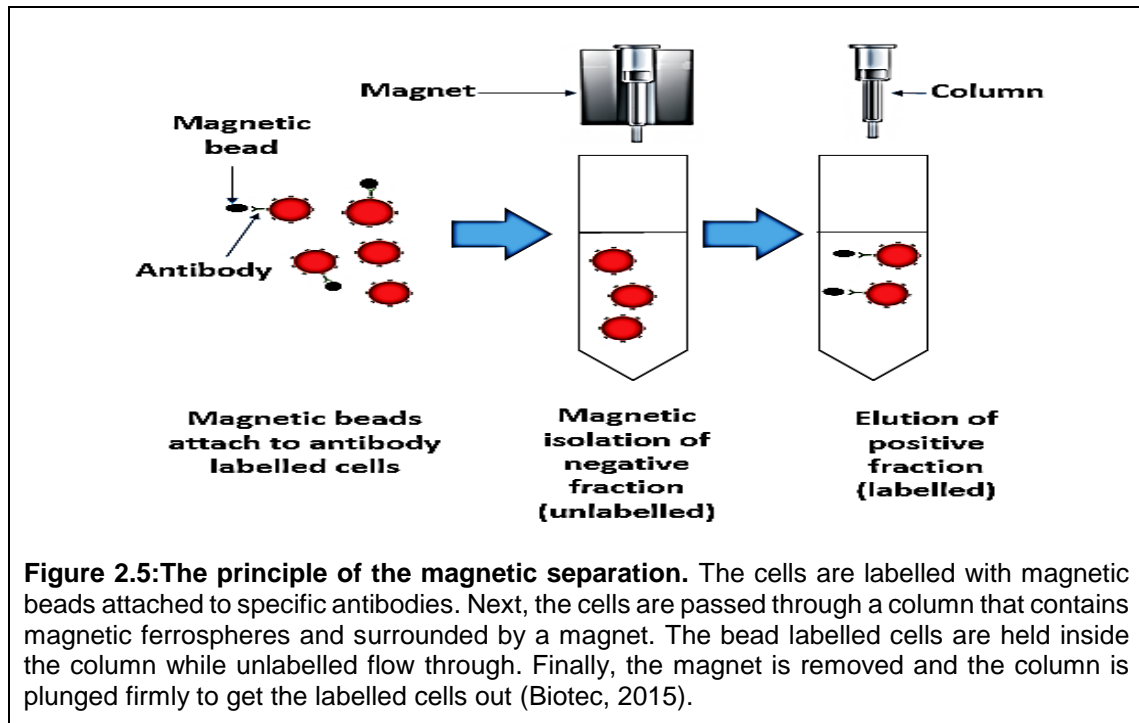
Requirements:

- 108 mL blood taken by venesection
- CD14, CD3, CD45RA, CD45RO microbeads
- Pan T Isolation kit
- LS columns
- MACS buffer: 50mls HBSS, 2mL (0.5M) EDTA, 2.5g BSA (mix and then filter 0.2µm). Then dilute with HBSS 10:1
- T-cell culture medium
- Human GM-CSF,
- Human IL-4
- Human TNFα
- LPS

Principles:

T-cell-DC priming: This assay is based upon the ability of the antigen presenting cells (in this assay DCs) to prime the naïve (CD45RO-ve) T-cells to become reactive (primed i.e. CD45RO+ve) to the drug under investigation. Successful priming can be achieved when DCs are incubated with the naïve cells at a ratio of 1:31 and the drug with a specific dose of the drug for 8 days. T-cells priming was performed according to the protocol described in (Faulkner et al., 2012).

Magnetic separation of cells: Magnetic bead labelled antibodies are prepared according to the type of cells that are required to be separated. These antibodies



will bind to specific receptor e.g. CD14 leaving the non-CD14 cells unlabelled. Then, the cells are poured in a column that contains magnetic ferrospheres and is surrounded by a strong mobile magnet. These ferrospheres amplify the magnetic field 10000 times and so creating a strong magnetic gradient to hold the labelled cells while allowing the flow of unlabelled cells (**Figure 2.5**). The spheres lie at 30 μM distance from each other which allows the separation of most kinds of cells. The labelled cells are not attached to the column matrix but they are rather held in suspension inside the column. After that, the magnet is taken off and the labelled cells are pushed out by plunging the column with MACS buffer (Biotec, 2015).

Procedure:

108 mL of blood was taken by the process of venesection from a naive volunteer and the blood cells were separated according to the protocol of the **heading 2.2.2**. All cell separations using the beads isolations mentioned below were carried out according to the protocols designed by manufacturer (Miltenyi Biotec Ltd).

2.3.1 Positive selection of CD14 cells (Day 0)

1. The cells, microbeads and MACS buffer should be all kept on ice 4°C.
2. The cells which were in AB medium were spun down. Then, (800 μ L) MACS buffer per 10^8 cells + (200 μ L) CD14 microbeads per 10^8 cells were added to the cells. After mixing, the mixture was incubated for 15 min in the fridge at 4°C.
3. MACS buffer (15 mL) per 10^8 cells were added to the mixture and then spun at 1500rpm for 10 min at 4°C.
4. The supernatant was pipetted and the cells were resuspended at 10^8 cells per (750 μ L) MACS buffer.
5. The LS column was placed on the magnet and washed with (3 mL) MACS buffer.
6. The cell suspension was added to the column. The tube was rinsed twice with (500 μ L) MACS buffer.
7. The column was washed 3 times with (3 mL) MACS buffer.
8. The flow through was collected as containing the CD14 –ve cells.
9. The column was removed from the magnet and placed over a collection tube. MACS buffer (5mL) was added to the column and the cells were harvested by applying the plunger firmly to the column. The collected cells are CD14 +ve cells.
10. By adding the MACS buffer (10 mL), the positive cells were made and counted by 1:2 dilutions, while the negative cells were made to (15 mL) and counted to 1:4 dilution.
11. The CD14 cells were resuspended at $12-16 \times 10^6$ cells/mL in 10% AB medium.
12. 0.5 mL of the cell suspension was used to establish the CD14 culture for the DCs, while the remaining CD14 cells were resuspended at $5-10 \times 10^6$ cells/cryovial, then 20% DMSO, 80% AB serum was added dropwise in an equal volume while the cryovials were still on ice 4°C. The cryovials were

stored overnight in Mr Frosty tubs at -80°C and then transferred to -150°C for storage.

13. The non-CD14 cells were used for T-cell separation.

2.3.2 Negative selection of CD3 T-cells (Day 0)

1. Non-CD14 were spun down and the supernatant was discarded. Then, (40µL) MACS buffer and (10µL) antibody cocktail per 10^7 cells were added to the cells. The cells were mixed well and incubated at 4°C for 10 min.
2. MACS buffer (30 mL) and (20 µL) anti-biotin microbeads per 10^7 cells were added to the cells. Following mixing, the cells were incubated at 4°C) for 15 min.
3. MACS buffer (1.5 mL) per 10^7 cells was added and spun at 1500 rpm for 10 min at 4°C. The supernatant was pipetted completely and resuspended at 10^8 cells in (500 µL) MACS buffer. Next, the cells were added to LS column. This was followed by twice rinsing of the tube with (500 µL) MACS buffer.
4. The LS column was washed with (3 mL) MACS buffer 3x.
5. The flow through was the CD3+ve cells. Their volume was made up to (15 mL).
6. The CD3-ve cells were recovered from the column and the volume was made up to (10 mL), then cells were counted.
7. CD3-ve cells were either frozen down or utilized in the preparation of EBVs according to the protocol discussed under the **heading 2.2.4**.

2.3.3 Positive selection of CD25 and CD45RO T-cells (Day 0)

1. The CD3 +ve cells were spun down and (70 µL) MACS buffer per 10^7 cells, (10 µL) CD25 and (20µL) CD45RO microbeads per 10^7 cells were added. After mixing, the cells were incubated for 15 min in the fridge at 4°C.

2. MACS buffer (1.5 mL) per 10^7 cells were added to the mixture. After centrifugation at 1500 rpm for 10 min at 4°C. The supernatant was removed completely and cells were resuspended at 10^8 cells per (500 μ L) MACS buffer.
3. The cells were added to the LS column, and then the tube was rinsed twice with (500 μ L) MACS buffer.
4. The LS column was washed with (3 mL) MACS buffer 3x.
5. The flow through cells (CD45RO–ve) were made up to (15 mL) by adding MACS buffer and counted at a 1:4 dilution. The harvested cells from the column by pressure (CD45RO +ve) were made up to (5 mL) and counted at 1:2 dilution.
6. The cells were cryopreserved.

2.3.4 DCs maturation from CD14 cells (Days 0, 2, 4, 6)

Principle: CD14 cells (monocytes) have the ability to differentiate into dendritic cells (DCs) in the presence IL-4, GM-CSF, LPS and TNF α (Han et al., 2009).

CD14 cells can differentiate into DCs or macrophages. The presence of IL-4 and GM-CSF favours the DC pathway and prevents the formation of macrophages, while TNF α and LPS promote the maturation of DCs (Hiasa et al., 2009).

1. (6 mL) of $1-2 \times 10^6$ CD14 cells/mL were suspended in medium containing (800 U/mL) GM-CSF and IL-4. Then, (3mL/well) were aliquoted to a 6 well plate.
2. On day 2, (3 mL/well) medium with (800 U/mL) GM-CSF and IL-4 were added.
3. On Day 4, (3 mL) of the medium supernatant/well were withdrawn and discarded. Then, 3 mL of the medium containing (800 U/mL) GM-CSF and IL-4 were added.

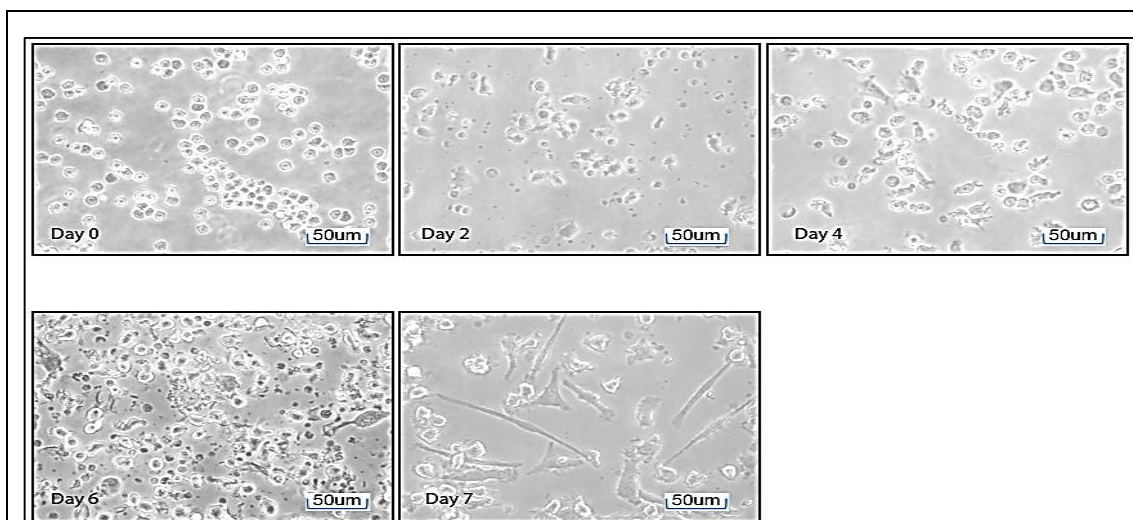


Figure 2.6: The stages of dendritic cells maturation from day 0 till day 7. The CD-14 cells on day 0 are round with no projections (dendrites). Day after day the number of the dendrites increases gradually and by day 7 the cells have long dendrites denoting that they are mature DCs.

4. On day 6, (3mL) of the supernatant/well were withdrawn and discarded. Then, 3mL/well medium containing (800 U/mL) GM-CSF and IL-4, (1 µg/mL) LPS and (25 ng/mL) TNFα were added. **Figure 2.6** shows the stages of dendritic cells maturation from day 0 to day 7

2.3.5 DCs stimulation of T-cells (coculture) (Day 7):

1. Frozen CD45RO-ve T-cells were thawed quickly and resuspended in medium. After washing, they were resuspended in medium and the count made up to 2.5×10^6 cells/mL in medium. Then, using 24 well plate, cells were added as (1mL/well).
2. DCs were harvested from the bottom of the plate using the scraper to wipe the bottom (3 times vertical and 3 times horizontal). Next, the cells were aspirated using a Pasteur pipette. Then, cells were spun down and resuspended in 10% AB medium at 1.6×10^5 cells/mL. (0.5 mL/well) was added to the wells of the 24 well plate containing T-cells.
3. The drug (piperacillin) was prepared in culture medium in a concentration that is 4 times the required concentration. (0.5 mL) of the prepared drug was added to the wells of the T-cell-DCs coculture. A control drug (SMX-

NO) was also prepared in the same way as piperacillin and added to one of the wells of the T-cell-DCs coculture.

4. The T-cell-DC coculture plate is then incubated at 37°C and 5% CO₂ for 8 days.

2.3.6 DCs culture from CD14 cells (Days 7, 9, 11, 14)

DCs were prepared using the same protocol mentioned under the **heading 2.3.4.**

2.3.7 TCR-V β analysis on day 7

Principle:

In order to measure the skewing of TCR-V β before and after the priming procedure, we analysed of the TCR-V β on day 7 (before the priming) and on day 18 (after the priming). The protocol for staining is mentioned below which entails in addition to the usual antibodies for the TCR-V β other antibodies for CD3 (general receptor for T-cells), CD4, CD8 (both for the phenotype of the T-cells), CD45RA and CD45RO (both to measure the magnitude of priming).

Procedure:

- **Flow cytometric analysis:**

1. (600 μ L) of naïve T-cells at 2×10^6 cells/ mL were set aside for the TCR-V β analysis.
2. (50 μ L) of cells were added to 12 non-sterile FACS tubes.
3. The antibodies CD3-APC, CD4-FITC, CD8-PE, CD45RO-PerCP-Cy5.5, CD45RA-FITC and TCR-V β antibodies A–H were added according to **Table 2.6.**
4. The tubes 1,2,11 and 12 were incubated on ice (at 4°C) while the tubes A–H were incubated at room temperature. All the tubes were kept in dark for 20 min.

5. (500 μ L) of FACS buffer was added to each tube. This was followed by centrifugation at 1500 rpm.
6. After discarding the supernatant, (200 μ L) FACS buffer was added to each tube.

Table 2.6: Fluorochrome antibodies that are used to stain the cells for FACS analysis.		
Tubes	Antibodies	TCR V β kit
1	None	None
2	CD3-APC (3 μ L), CD45RO (3 μ L)	None
3	CD3-APC (3 μ L), CD45RO (3 μ L)	A (5 μ L)
4	CD3-APC (3 μ L), CD45RO (3 μ L)	B (5 μ L)
5	CD3-APC (3 μ L), CD45RO (3 μ L)	C (5 μ L)
6	CD3-APC (3 μ L), CD45RO (3 μ L)	D (5 μ L)
7	CD3-APC (3 μ L), CD45RO (3 μ L)	E (5 μ L)
8	CD3-APC (3 μ L), CD45RO (3 μ L)	F (5 μ L)
9	CD3-APC (3 μ L), CD45RO (3 μ L)	G (5 μ L)
10	CD3-APC (3 μ L), CD45RO (3 μ L)	H (5 μ L)
11	CD3-APC(3 μ L), CD4-FITC (3 μ L), CD8-PE(3 μ L) CD45RO(3 μ L)	None
12	CD3-APC (3 μ L), CD45RA(10 μ L), CD45RO (3 μ L),	None

7. The compensation was prepared according to the protocol mentioned under the **heading 2.2.7** using the antibodies for CD3-APC, CD4-FITC, CD8-PE and CD45RO-PERCP-CY5.5
8. The cells were analyzed using FACS Canto II. At least 50000 cells per tube were acquired. Cells percentages were analyzed later by Cyflogic software.

2.3.8 T-cells reactivity and specificity assays (Day 15)

Principle:

The aim of these assays is to check the efficiency of the priming in terms of sensitivity and specificity. This can be accomplished by implementing several

useful assays which include: ELISPOT, ^3H -thymidine uptake proliferation assay, CFSE uptake analysis and chemokine receptor analysis.

^3H -thymidine uptake proliferation assay involves culturing primed T-cells and DCs with the culprit drug for 48-72 hrs.

From previous research on priming naïve T-cells from naïve healthy volunteers, it was found that the DC-T-cell coculture post priming does not contain many primed T-cells as what is found in TCCs. Therefore, it was decided to make the duration of ^3H -thymidine uptake proliferation assay 72 hrs instead of 48 hrs to allow more time for the activation of drug specific T-cells to achieve higher counts to be detected by beta counter.

Procedure:

DCs and the T-cells were harvested according to the protocol mentioned under the **heading 2.3.5**. The T-cells which were in two groups: one primed for piperacillin and the other is primed for SMX-NO. Both were made up to 2×10^6 cells/mL. (50 μL /well) was added into U-bottom 96 well plate. The DCs were made up to 1.6×10^5 cells/mL and (25 μL /well) was added.

2.3.8.1 ELISPOT

1. The plate for ELISPOT was coated the day before with coating antibodies for IFN- γ , IL-5 and IL-13 according to the protocol mentioned under the **heading 2.2.6**. T-cells and the DCs and the drugs were added according to the conditions and doses mentioned in **Table 2.7**. The drugs were added in (50 μL). Then, all the wells were made up to 200 μL by adding medium.
2. The processing of the ELISPOT plate was conducted on day 17 of the T-cell priming assay according to the protocol mentioned under the **heading 2.2.6**.

Table 2.7: The design of the ELISPOT plate in terms of cells and the doses of the drugs used.

Piperacillin primed cells IFN- γ		Piperacillin primed cells IL-5		Piperacillin primed cells IL-13		SMX-NO primed cells IFN- γ		SMX-NO primed cells IL-5		SMX-NO primed cells IL-13	
0	0	0	0	0	0	0	0	0	0	0	0
0.5mM pip	0.5mM pip	0.5mM pip	0.5mM pip	0.5mM pip	0.5mM pip	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO
1 mM pip	1 mM pip	1 mM pip	1 mM pip	1 mM pip	1 mM pip	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA
2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip
5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA	5 μ g/mL PHA						
50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO	50 μ M SMX-NO						

2.3.8.2 ^3H -thymidine uptake proliferation assay:

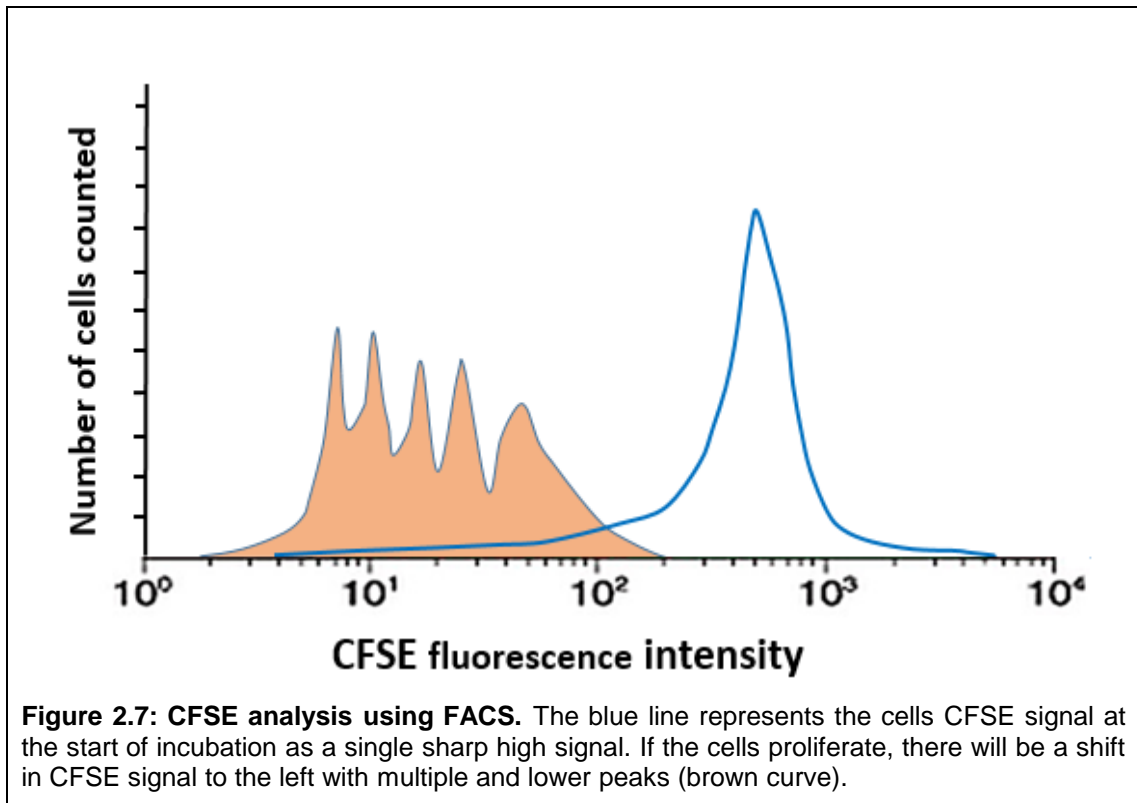
1. The cells were added to the plate using the same counts and volumes mentioned in the **heading 2.3.8.1** with the exception that they were arranged in triplicates as shown in **Table 2.8**. In addition to that, there are wells for T-cells only, DCs only and T-cells+DCs+ (5 μ g/mL) PHA as control conditions.
2. ^3H -thymidine was added on day 18 of the T-cell priming assay (i.e. after 72 hrs of the preparation of the test plate). After 16 hrs of incubation, the plate was harvested according to the protocol mentioned under the **heading 2.2.5.4**.

Table 2.8: design of the ^3H -thymidine uptake proliferation assay plate in terms of conditions and cells.

Piperacillin primed cells			Piperacillin primed cells				SMX-NO primed cells		
0	0	0	DCs (only)	DCs (only)	DCs (only)		0	0	0
0.5 mM pip	0.5 mM pip	0.5 mM pip	T-cells (only)	T-cells (only)	T-cells (only)		50 μM SMX-NO	50 μM SMX-NO	50 μM SMX-NO
1 mM pip	1 mM pip	1 mM pip	5 $\mu\text{g/mL}$ PHA	5 $\mu\text{g/mL}$ PHA	5 $\mu\text{g/mL}$ PHA		2 mM pip	2 mM pip	2 mM pip
2 mM pip	2 mM pip	2 mM pip							
50 μM SMX-NO	50 μM SMX-NO	50 μM SMX-NO							

2.3.8.3 Proliferation assay by CFSE (Carboxyfluorescein succinimidyl ester) uptake:

Principle: CFSE is an intracellular fluorescent stain with a fluorochrome wavelength in the range of the FITC stain. It binds to intracellular molecules especially lysine residues by forming covalent bonds with the privilege to be retained in cell for very long time (Parish, 1999). CFSE has been used extensively to measure cellular proliferation by flow cytometry. CFSE-labelled cells proliferation will lead to spread of the CFSE stain on to a greater number of cells (daughter cells) (Lyons et al., 2013) which appear on the histogram of the FACS Canto II as a shift of the curve to the left with diminished peak (**Figure 2.7**). This technique if combined with the labelling of the cells with specific fluorochrome antibodies (e.g. CD4-APC, CD8-PE) has the advantage over thymidine uptake proliferation assay that it can measure specific cell subset proliferation rates (Hilchey and Bernstein, 2007).

**Procedure:****• CFSE staining:**

1. The cells were transferred to sterile FACS tubes (one tube for the cells primed to piperacillin and the other for cells primed with SMX-NO).
2. The cells were spun down at 1500 rpm for 5 min and the supernatant was discarded. Then, the cells were resuspended in (1 mL) 5% AB/HBSS.
3. The wash process was repeated as above.
4. CFSE (2 μ L) was added to Eppendorf vial that contained (100 μ L) HBSS
5. CFSE mixture (50 μ L) was added to each tube of cells. The tubes were protected from light and incubated at room temperature for 5 min.
6. (1 mL) 5% AB/HBSS was added per tube and then the tubes were spun at 1500 rpm for 5 min and the supernatant was discarded. The cells were then resuspended in 1 mL 5% AB/HBSS.

7. The wash process was repeated as above.
8. Medium was added to the cells which were resuspended at (2×10^6 cells/mL).
9. Using the same counts and volumes mentioned in the **heading 2.3.8.1**. The T-cells, DCs and drugs were added to the wells of the plate shown in the **Table 2.9**.

- **CFSE analysis (Day 19):**

1. (90 μ L) of the wells supernatant were removed from each well of the CFSE plate and duplicate wells were combined.
2. (3 μ L) of antibodies CD4-APC, CD8-PE were added to each well with the exception of the CFSE only labelled and the unlabelled cell conditions.
3. Compensation controls were prepared using beads which were labelled with the CD4-APC, CD8-PE and CD3-FITC antibodies.
4. The cells were incubated on ice for 20 min.
5. (200 μ L) of FACS buffer was added to each well and then the cells were spun for 3 min at 2200 rpm at 4°C. The supernatant was discarded.
6. The cells were resuspended in (200 μ L) FACS buffer in preparation for the FACS analysis and transferred to tubes. At least 50000 cells/tube were acquired by the FACS Canto II, then, the results were analyzed by the aid of Cyflogic software. The cells were first gated on FSC (forward scattering) and SSC (side scattering) to identify the lymphocyte population. Then, the lymphocyte population was gated for CD4 or CD8 population. The amount of CFSE was plotted as a histogram.

Table 2.9. Design of the CFSE assay plate in terms of conditions and cells.					
Piperacillin primed cells		Piperacillin primed cells		SMX-NO primed cells	
0	0	Unlabelled cells	Unlabelled cells	0	0
0.25mM pip	0.25mM pip	Labelled cells	Labelled cells	25 μ M SMX-NO	25 μ M SMX-NO
0.5mM pip	0.5mM pip			50 μ M SMX-NO	50 μ M SMX-NO
1 mM pip	1 mM pip			5 μ g/mL PHA	5 μ g/mL PHA
2 mM pip	2 mM pip			2 mM pip	2 mM pip
5 μ g/mL PHA	5 μ g/mL PHA				
50 μ M SMX-NO	50 μ M SMX-NO				

2.3.8.4 Chemokine receptor analysis:

Principle: The assay focuses on the chemokine receptor expression of the primed cells. This is achieved by labelling the cells with CD45RO and the chemokine receptor antibodies. Then, the gating is based upon the CD45RO and the chemokine receptor expression by which the CD45RO positive and negative cells are compared and analyzed with the aid of FACS Canto II (Faulkner et al., 2012).

Procedure:

The plate was prepared (on day 15) using the same counts and volumes mentioned under the **heading 2.3.8.1**.

1. The wells supernatant (90 μ L) were removed from each well of the plate (**Table 2.10**) and duplicate wells were combined (so instead of 14 wells we have 7 wells, each one of them would be stained as one of the 7 tubes used in the assay).

Table 2.10: Design of the chemokine receptor expression assay plate in terms of conditions and cells.											
Piperacillin primed cells											
2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip	2 mM pip
2 mM pip	2 mM pip										

- (3 μ L/well) of antibodies was added according to the **Table 2.11**. The cells were incubated on ice for 20 min.
- Compensation controls were prepared using beads which were labelled with the CD45RO-Cy5 (2 μ L), APC (3 μ L), PE (3 μ L), FITC (3 μ L)
- (200 μ L/well) FACS buffer was added and the plate was spun for 3 min at 2200 rpm at 4°C. The supernatant was discarded.
- (100 μ L/well) FACS buffer was added. Then, the contents were transferred to non-sterile FACS tubes.
- The cells were analyzed by flow cytometric analysis. At least 50000 cells per tube were acquired. Mean fluorescence index of the cells was analyzed by Cyflogic software.

Table 2.11 Staining of chemokine receptor expression tubes				
Tube	Cy5.5	APC	FITC	PE
1	none	none	none	none
2	CD45RO 2 μ L	none	none	none
3	CD45RO 2 μ L	CXCR3 3 μ L	CCR5 3 μ L	CCR1 3 μ L
4	CD45RO 2 μ L	CCR2 3 μ L	CCR3 3 μ L	CCR4 3 μ L
5	CD45RO 2 μ L	CCR9 3 μ L	CCR8 3 μ L	CCR10 3 μ L
6	CD45RO 2 μ L	CCR6 3 μ L	CLA 3 μ L	CXCR6 3 μ L
7	CD45RO 2 μ L		CD69 3 μ L	E-cad 3 μ L

2.4 Identifying optimal dose of the drugs for T-cells priming (toxicity assay)

Requirements:

- Volunteers PBMCs
- Medium
- PHA
- Culprit drug

Principle:

Each drug has an optimal dose for which T-cells are the most reactive in terms of the priming assay. Higher doses than optimal would lead to toxicity and T-cells death. While lower doses than optimal wouldn't yield positive results even though the T-cells are sensitive.

The toxicity assay involves incubating PBMCs from a volunteer with serially diluted doses of the drug in the presence and absence of (5µg/mL) PHA.

The optimal dose is selected when the PHA stimulated proliferation curve starts to decrease (in terms of cpm proliferation). Only culprit drug doses that do not inhibit the T-cells proliferation by more than 15% can be utilized in priming assays (Pichler and Tilch, 2004).

Table 2.12: Design of the plate for the toxicity assay in terms of drugs, conditions and doses. 100000 PBMCs are incubated in each well (in triplicates) in addition to serially diluted doses of the culprit drug with and without 5µg/ml PHA.

Culprit drug + 5µg/ml PHA	Culprit drug
64x	64x
32x	32x
16x	16x
8x	8x
4x	4x
2x	2x
1x	1x
0	0

Rarely, the culprit drug could be stimulatory for PBMCs proliferation, a situation which denotes that optimal dose would be identified from (PHA -ve) conditions. This was not seen during this piece of work.

Procedure:

Volunteers PBMCs were incubated at 100000/well in triplicates in 96-well U bottomed plate in two conditions: in the presence and absence of (5 µg/mL) PHA (**Table 2.12**). Serially diluted doses of the drug are added to the wells making the total volume of the well (200 µL). The cells were incubated for 24 hrs at 37°C and 5% CO₂. Then, for the final 16 hrs (0.5 µCi) of ³H-thymidine/well was added that is followed by harvesting and reading the plate using beta counter.

2.5 PBMCs priming

Principle:

PBMCs typically contain APCs which could be macrophages, B-cells, DCs in addition to the presence of naïve and memory T-cells. Hence, incubating the PBMCs with culprit drugs would provide the chance to generate primed T-cells to the culprit drug via priming naïve T-cells.

Another possibility, PBMCs may already contain some drug specific memory T-cells. Thus, by incubating PBMCs with this drug, the APCs would have the chance to provide stimulatory signals to trigger the proliferation of these cells boosting their numbers. Repeating this incubation process several times should boost the numbers of the primed T-cells to a level that may yield positive results in terms of reactivity to culprit drug in in vitro T-cells assays.

Procedure:

1. PBMCs are collected from a donor and counted to make up ($4-6 \times 10^6$ cells/mL) in medium. Then, the cells were aliquoted into 24-well plate as (1mL/well).
2. Culprit drug was prepared at 2x final concentration then; it was added to the test plate as (1mL/well).
3. SMX-NO wells are prepared as an irrelevant drug antigen control.
4. The plate was cultured at 37°C and 5% CO₂ for 5 days.
5. From day 5, the wells were fed by withdrawing (600µL) and adding (660µL) of medium containing (2µL/mL) IL-2 every 2-3 days.
6. Every two weeks (day 14, 28) the PBMCs cultures were restimulated with 1mL irradiated autologous PBMCs (1.5×10^6 cells/mL) with drugs 2x + (200 U/mL) IL-2.
7. Autologous EBVs were generated for each donor.
8. T-cells assays responses were carried out on days 14/28/35 which include ³H- thymidine proliferation assay & IFN-γ ELISPOT.
9. Clones were generated from the PBMCs using serial dilution technique.

2.6 Liquid chromatography/Tandem mass spectrometry

Principle:

Over the last two decades there was a revolutionary progress in inventing and developing the technique of mass spectrometry (MS) which paved the way for

more detailed and sophisticated analytic studies especially in the field of proteomics.

MS instruments that are utilising electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) are combined with other mass analysers (time-of-flight (TOF), quadrupole or ion trap) to create a highly sensitive and specific tool for protein identification (Ahmed, 2008;Koomen et al., 2005;Patterson SD, 2001).

The first stage in processing the sample involves **protein(s) cleavage** using different reduction and alkylation steps in which the disulphide bonds cross linking the protein are broken and capped to prevent their reestablishment.

This process involves unfolding the protein to allow the enzyme (trypsin) get access to the polypeptide backbone. This process results in a series of peptides.

In **liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS)**, the peptides are prefractionated using inline reversed phase chromatography, thus the MS machine will analyse few species at a time.

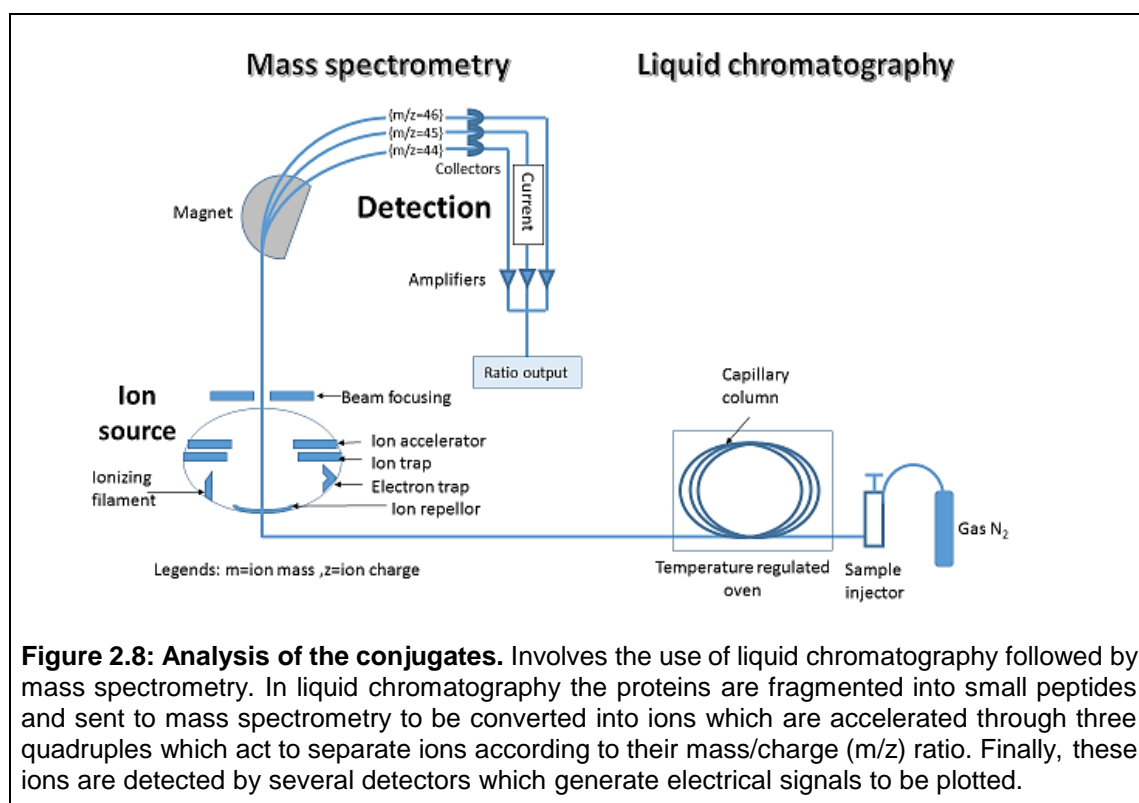
Every multiple charged peptide ion will be bombarded by with nitrogen leading to a reasonable fragmentation at the peptide bond between the amino acid residues. In this way the LC-MS/MS can yield precise data about the sequence and the chemical, post-translational modifications of the proteins.

This highly sensitive technique analyses molecules depending on their weight (mass, m) and the charge (z). In general, the mass spectrometer is composed of three components which are the **ion source**, **mass analyser** and the **ion detector (Figure 2.8)**.

The sample in our study is composed of a complex mixture of peptides. Initially, the sample will undergo vaporization and ionization process which is followed by accelerating the ions. The ions are selected according to their mass to charge

ratio (m/z) and then undergo further fragmentation. This is followed by the second round of MS in which the m/z of the fragments is determined.

Multiple Reaction Monitoring MS (MRM-MS) is another highly sensitive and specific method for detecting and quantifying peptides in complex mixtures (Kitteringham et al., 2009). Here a triple quadrupole instrument is essential since it is able to select ions of specific m/z ratio in the first Q1 and the third quadrupole Q3 by modifying the voltage settings. Quadrupole 2 (Q2) functions as a collision chamber for the peptides to be fragmented into smaller ions to reveal the peptide sequences.



Consequently, the ions are selected in Q1 and fragmented in Q2 and then preferentially selected in Q3 (Unwin et al., 2009).

The transition (combination of parent ion mass and fragment ion mass) is designed to be as diagnostic for the peptide of interest as possible, but it also

should be detected with good sensitivity. This means that the selection of the MRM transitions is critical for highly sensitive and specific peptide quantification.

Procedure:

HSA-drug conjugates were reduced by the addition of 10mM dithiothreitol (DTT) followed by incubation room temperature for 15 minutes. The cysteine residues were capped by the addition of 55mM iodoacetamide and incubation for a further 15 minutes at room temperature. The protein solution was diluted to (3.2mg/mL) in phosphate buffer and to each 50 μ L (160 μ g) aliquot was added 5 μ g sequencing-grade modified trypsin. The samples were incubated overnight at 37°C. Then, they were desalted by reversed phase chromatography using C₁₈ ZipTips and dried in a centrifugal concentrator.

Samples were reconstituted in 2% acetonitrile (ACN)/0.1% formic acid (v/v), and aliquots of (2.4 – 5 pmol) were delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer by automated in-line LC (U3000 HPLC System, 5 mm C₁₈ nano-precolum and 75 μ m \times 15 cm C₁₈ PepMap column) via a 10- μ m inner diameter PicoTip. A gradient from 2% ACN/0.1% formic acid (v/v) to 50% ACN/0.1% formic acid (v/v) in 70 min was applied at a flow rate of 300 nL/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 18, and the interface heater to 150°C. Multiple reaction monitoring (MRM) transitions specific for drug-modified peptides were selected as follows: the mass/charge ratio (m/z) values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the appropriate hapten (cyclized Piperacillin, 517 atomic mass units [amu]; hydrolyzed Piperacillin, 535 amu; Amoxicillin, 365 amu; Amoxicillin with loss of NH₂, 349 amu; Benzyl Penicillin, 334 amu; Flucloxacillin, 453 amu; Penicillin V, 350 amu); the parent ion masses were then paired with a fragment mass of 160 ([M+H]⁺ of cleaved thiazolidine ring present in all of the haptens) and/or a fragment mass of 106 ([M+H]⁺ of cleaved benzylamine group of hydrolyzed Piperacillin haptens). MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles in order to

maximize specificity, they were optimized for collision energy and collision cell exit potential, and the dwell time was 50 ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample as analyzed by conventional LC tandem MS and were used to normalize sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (Sciex). Epitope profiles were constructed by comparing the relative intensity of MRM peaks for each of the modified lysine residues within a sample and normalization of those signals across samples.

The analysis of piperacillin-HSA conjugates was performed by exploiting a very characteristic fragment ion from the drug hapten. The thiazolidine ring of the drug was readily cleaved leading to an intense ion in the MS/MS spectrum at m/z 160 (Jenkins et al., 2009). The presence of the drug also hindered access by trypsin to the peptide backbone of the protein, leading to a missed cleavage at every modified lysine residue. The masses selected in Q1 were therefore the theoretical masses of every peptide in a particular protein with a missed cleavage at lysine plus the mass of the drug (+517 for the cyclized, +535 for the hydrolyzed, +489 for the desethyl cyclized and +507 for the desethyl hydrolyzed hapten). The fragment ion selected in Q3 was 160. Using these “transitions” highly sensitive and selective identification of modified peptides could be achieved.

2.7 HSA conjugate western blots

Aliquots of 1 μ g protein conjugate were prepared in Laemmli sample buffer and electrophoresed on 10% SDS-PAGE minigels. The proteins were transferred to a nitrocellulose membrane and blocked with 2% non-fat dry milk block in Tris/saline/Tween (150mM NaCl; 10mM Tris-HCl, pH 8.0; 0.1% Tween-20) overnight at 4°C. Tris/saline/Tween was used to perform all washes. Anti-penicillin primary antibody was diluted 1:20000 in 2% skimmed milk/TST and incubated for

1h at room temperature with shaking. The blots were washed for 3 X 5mins in TST before incubation with secondary goat anti-mouse HRP conjugated antibody, 1:2000 in 2% skimmed milk/TST for 1h at room temperature. Bands were detected by incubation with Western Lightning Chemiluminescence reagent for 1min. This was followed by exposure to ECL sensitive film and imaging on a GS-800 densitometer.

2.8 Synthesis of drug-modified albumin conjugates

Synthetic drug HSA conjugates were generated for functional studies by incubating drugs (piperacillin, penicillin G, and amoxicillin) with HSA at molar ratio of 100:1-250:1 for 24hr in phosphate buffer. The conjugates were purified by ultracentrifuge with a 3,000 MW cut off centrifugal filter according to the manufactory's protocol. Briefly, 600 μ L conjugates were added to the filter device followed by addition of 14 mL phosphate buffer (10mM, pH 7.4). The device was centrifuged at 5000Xg for 20 min and the filtrate was discarded. The process of washing was repeated for 6 times until the concentration of the free drug has been sufficiently reduced. The concentration of free drug in the filtrate from the last wash was determined by LC-MS and was found to be below 2.5nM.

2.9 Isolation of HSA from plasma

HSA was isolated by affinity chromatography, A POROS anti-HSA affinity cartridge attached to a Vision Workstation was used to affinity capture HSA which was then eluted with 12 mM hydrochloric acid. Fractions containing protein were identified by means of UV detection at 280 nm, and were methanol precipitated and processed for MS analysis, as described previously.

2.10 Ethics approval

The piperacillin hypersensitive patients with cystic fibrosis enrolled in the study on the piperacillin from the Regional Adult Cystic Fibrosis Unit, St James's University

Hospital, and Leeds, UK. The parameters of diagnosing CF were simply typical clinical profile in addition to positive sweat test and/or mutations involving both alleles of the cystic fibrosis transmembrane conductance regulator gene.

Piperacillin hypersensitive patients were defined as a patient who developed hypersensitivity reaction after 48 hrs or more of I.V. piperacillin administration

The naive group were healthy volunteers who have not been administered the tested antibiotic/drug.

Written informed consent was obtained from all the patients and the volunteers. Moreover, the study was granted approval by the Leeds East Ethics Committee.

For healthy volunteers in the telaprevir chapter the approval was obtained from the Liverpool Local Research Ethics Committee. Informed written consent was obtained from each volunteer.

2.11 Statistical Analysis

Statistical analysis was carried out by implementing the Kruskal-Wallis test in case of many conditions (three or more) and using Dunn's test for multiple comparisons. When there were two conditions per experiment, the statistical analysis was performed utilising Mann–Whitney test.

The implemented level of statistical significance for both tests is $p < 0.05$. The levels of significance are denoted as *, ** and *** where they were positive which signifies 0.05, 0.01 and 0.001 respectively. The error bars wherever found in figures represents standard deviation.

The graphs were drawn using Graph Pad Prism 5 software apart from pie charts and the graphs of mass spectrometry which were conducted using Excel.

Chapter Three

**Free
Piperacillin**

3 Characterization of the specificity and functionality of drug-specific T-cells isolated from piperacillin hypersensitive patients with cystic fibrosis

3.1 Introduction

Over the last decade, there has been great focus on the field of personalized medicine trying to tailor medical services (in terms of decision, clinical practice, diagnostic tests and therapies) to suit each patient individually. This was the result of and at the same time the cause for the development of revolutionary diagnostic techniques capable of delineating the patients into categories that are defined by specific criteria which eventually qualify them to personalized therapeutic modalities.

Specific HLA alleles are now known to be associated with increased susceptibility to different forms of drug hypersensitivity. However, this is not the case for piperacillin. The high frequency of reactions excludes the involvement of a single allele.

One alternative aspect of this rapidly growing field is the importance placed on T-cell phenotype. Several researchers have recently focused on the possible involvement of T-cells expressing a specific TCR-V β phenotype. In fact, it has been suggested that TCR-V β analysis may be used for both the screening of susceptible individuals and the treatment of patients with drug hypersensitivity reactions (Ko et al., 2011).

Thus, we have conducted research in this area trying to identify a possible pathognomonic TCR-V β profile for susceptible candidates for developing piperacillin hypersensitivity.

Consequently, we have addressed the following questions:

1. Is it possible to generate TCCs to free piperacillin from patients PBMCs?
2. Is there a predominant pattern for TCR-V β expression in piperacillin-specific TCCs?
3. Is it possible to prime healthy volunteer T-cells to free piperacillin? If so, what is the TCR-V β profile after priming and is it similar to that of the patients' TCCs?
4. Is it possible to generate clones to free piperacillin from the primed T-cells of healthy volunteers? What is their TCR-V β expression profile? Is it similar to that of the patients' TCCs or the primed T-cells from the T-cells priming assays?
5. What is the sensitivity, specificity, and cytokine secretion profile and chemokines receptor pattern on piperacillin-specific clones?

3.2 Aim of the study

In accordance with the questions above, we have formulated the main aim of this chapter to be:

- To explore T-cell antigenic determinants in hypersensitive patients and healthy volunteers and the nature of the induced response focussing on TCR V β usage and cytokine release.

3.3 Materials & methods

The methods implemented in this chapter are described in detail in the relevant sections in materials and methods chapter and they are shown in **Figure 3.1**, **Figure 3.12** and **Figure 3.22**.

3.4 Results:

Notes:

- The term **selected clone** means selecting a well growing clone from serial dilution plates.
- The term **reactive clone** implies a clone that achieves an SI \geq 2 unless mentioned otherwise. I.e. it is synonymous with **sensitive clone**.
- The term **Bulks** implies T-cells or PBMCs post priming either by (DC-T-cell priming technique or by incubating PBMCs with culprit drug respectively).
- **Statistical significance** is mentioned whenever it is confirmed.
- The term **free piperacillin** is piperacillin that comes commercially in a powder form and dissolved in medium to be added to culture plates instantaneously without any intermediate processing as opposed to **conjugated piperacillin** which requires processing to be generated.

3.4.1 Piperacillin reactive clones from piperacillin hypersensitive patients

3.4.1.1 Generation of piperacillin reactive clones from piperacillin hypersensitive patients

Free piperacillin specific and sensitive clones were generated and tested for phenotype, TCR-V β , chemokine receptor expression and cytokine secretion according to the scheme shown in **Figure 3.1**.

PBMCs were collected from six CF piperacillin hypersensitive patients who have previously shown to be +ve for piperacillin hypersensitivity in LTTs with historical

delayed-onset maculopapular exanthema (Whitaker et al., 2011). In every case, the treatment had to be discontinued, and patients received anti-histamines and/or oral steroids, when clinically indicated. Blood samples were collected after 4 weeks. The demographics of those patients are illustrated in **Table 3.1**.

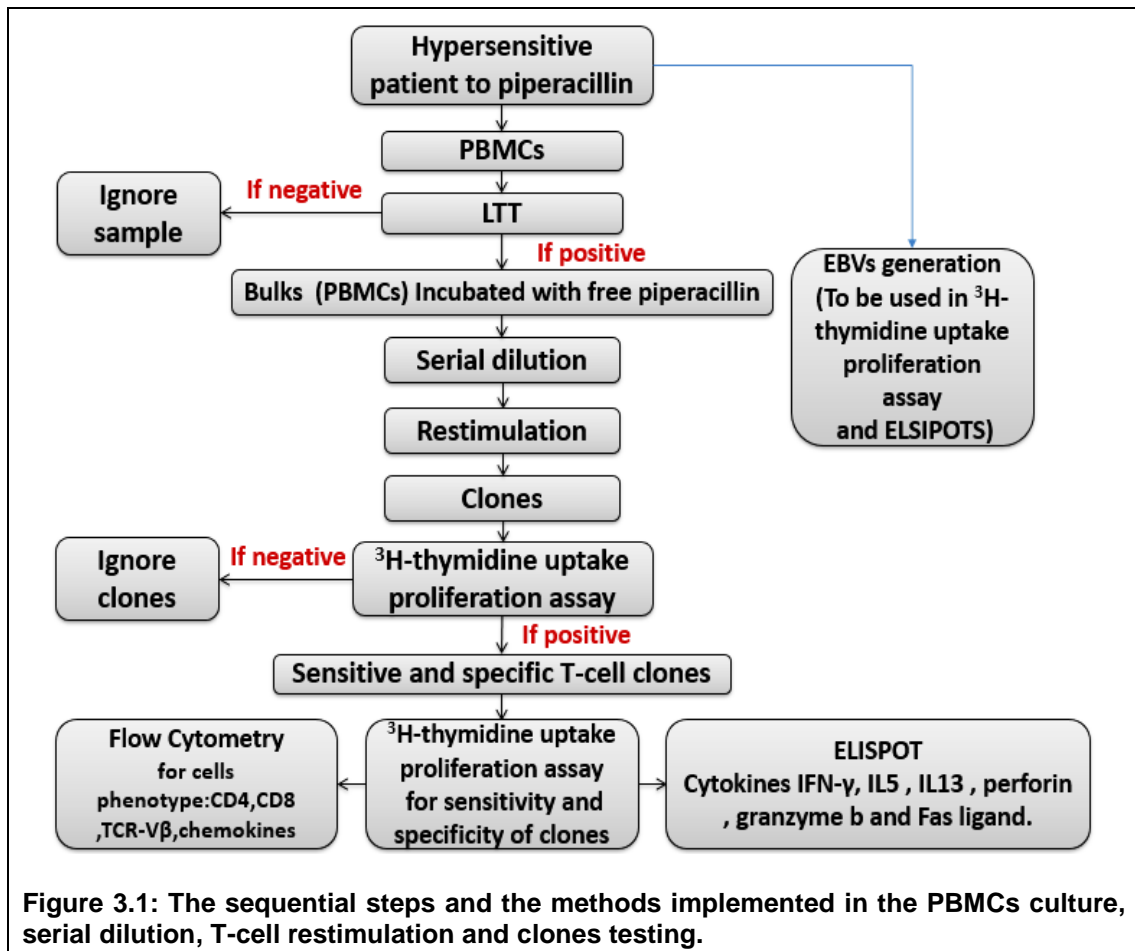
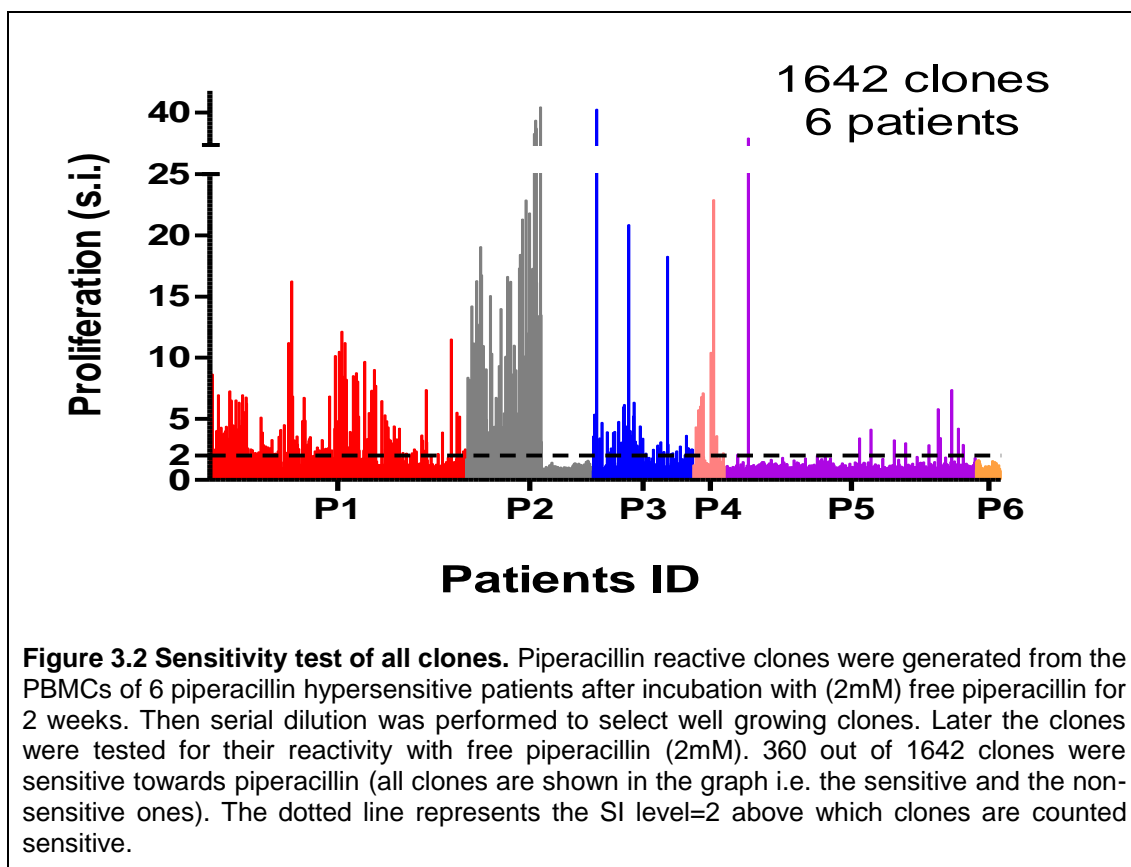


Figure 3.1: The sequential steps and the methods implemented in the PBMCs culture, serial dilution, T-cell restimulation and clones testing.

Table 3.1: Demographic data of the piperacillin hypersensitive CF patients.

Patient	Age/ Gender	Clinical features	Drug	Reaction	Delay between course initiation and reaction (days)	Time since reaction occurred (years)	Skin Prick Test	Delayed intradermal readings
P1	27 M	Cystic Fibrosis Chronic Pseudomonas infection FEV1 1.1lites (28% predicted) BMI 17 Osteoporosis 96 days intravenous antibiotics over past 12 months	Ceftazidime Piperacillin	MPE MPE/ fever	5 2	4 0.5	-ve -ve	-ve +ve at 48 hours
P2	26 M	Cystic Fibrosis Chronic Pseudomonas infection FEV1 1.82 lites (48% predicted) BMI 32 CF related diabetes 48 days of intravenous antibiotics over past 12 months	Ceftazidime Piperacillin	Fevers MPE	2 5	4 2	-ve -ve	-ve +ve at 48 hours
P3	21 F	Cystic Fibrosis Chronic Pseudomonas infection FEV1 0.63 lites (19% predicted) BMI 17 Osteoporosis 84 days of intravenous antibiotics over past 12 months	Piperacillin Ceftazidime	Arthralgia /fevers MPE	12 6	1 5	Not done Not done	Not done Not done
P4	26 M	Cystic Fibrosis Chronic Pseudomonas infection FEV1 2.87 litres (68% predicted) BMI 23 CF related diabetes 5 days of intravenous antibiotics over last 12 months	Piperacillin Aztreonam Ceftazidime Meropenem	MPE/ Fevers MPE Delayed Angio- edema MPE	9 4 3 5	7 5 5 5	-ve -ve -ve -ve	-ve -ve -ve -ve
P5	35 F	Cystic Fibrosis Chronic Pseudomonas infection FEV1 1.91 lites (62% predicted) BMI 25 35 days of intravenous antibiotics over past 12 months	Ceftazidime Piperacillin	MPE Fevers/ arthralgia	8 9	4 6	-ve -ve	-ve -ve
P6	28 M	Cystic Fibrosis Chronic Pseudomonas infection FEV1 0.61lites (15% predicted) BMI 16 Osteoporosis 197 days of intravenous antibiotics over past 12 months	Piperacillin	MPE	11	4	-ve	+ve at 24 hours



Clones were generated by serial dilution and tested using a lymphocyte proliferation assay. If clones were responsive to free piperacillin at (2mM) (i.e. $SI \geq 2$ to indicate a reactive clone), they were expanded by mitogen stimulation and characterized in greater detail. In total, six patients yielded 360 piperacillin reactive clones (out of 1642) (**Figure 3.2**). The number of piperacillin reactive clones generated from each patient was highly variable. Five patients yielded many reactive clones while one patient did not yield any (**Table 3.2**).

Table 3.2: Free piperacillin clones generated from six piperacillin hypersensitive CF patients. PBMCs were separated from their blood and bulks were prepared followed by serial dilution. The clones were tested at (2mM) free piperacillin. $SI \geq 2$ was the cut-off point to identify sensitive clones.			
Patient I.D number	Number of reactive clones	Total clones number	Percentage of reactive clones
P 1	193	533	36.2%
P 2	92	262	35.1%
P 3	53	208	25.5%
P 4	10	69	14.5%
P 5	12	517	2.3%
P 6	0	53	0.0%
Total	360	1642	21.9%

3.4.1.2 Quantitative assessment of the piperacillin clones reactivity to piperacillin

195 piperacillin reactive clones from 5 patients were challenged with serially diluted doses of piperacillin starting with (2mM) in addition to the medium as a negative control.

Clones displayed a dose-dependent response to piperacillin with the maximal response observed with (2mM) free piperacillin with high statistical significance at $p < 0.001$ (as analyzed by Kruskal-Wallis test as shown in) (**Figure 3.3**).

Figure 3.4 shows the different dose dependent proliferative responses observed with selected piperacillin-responsive clones. For all clones, the strongest response was observed with 2mM piperacillin.

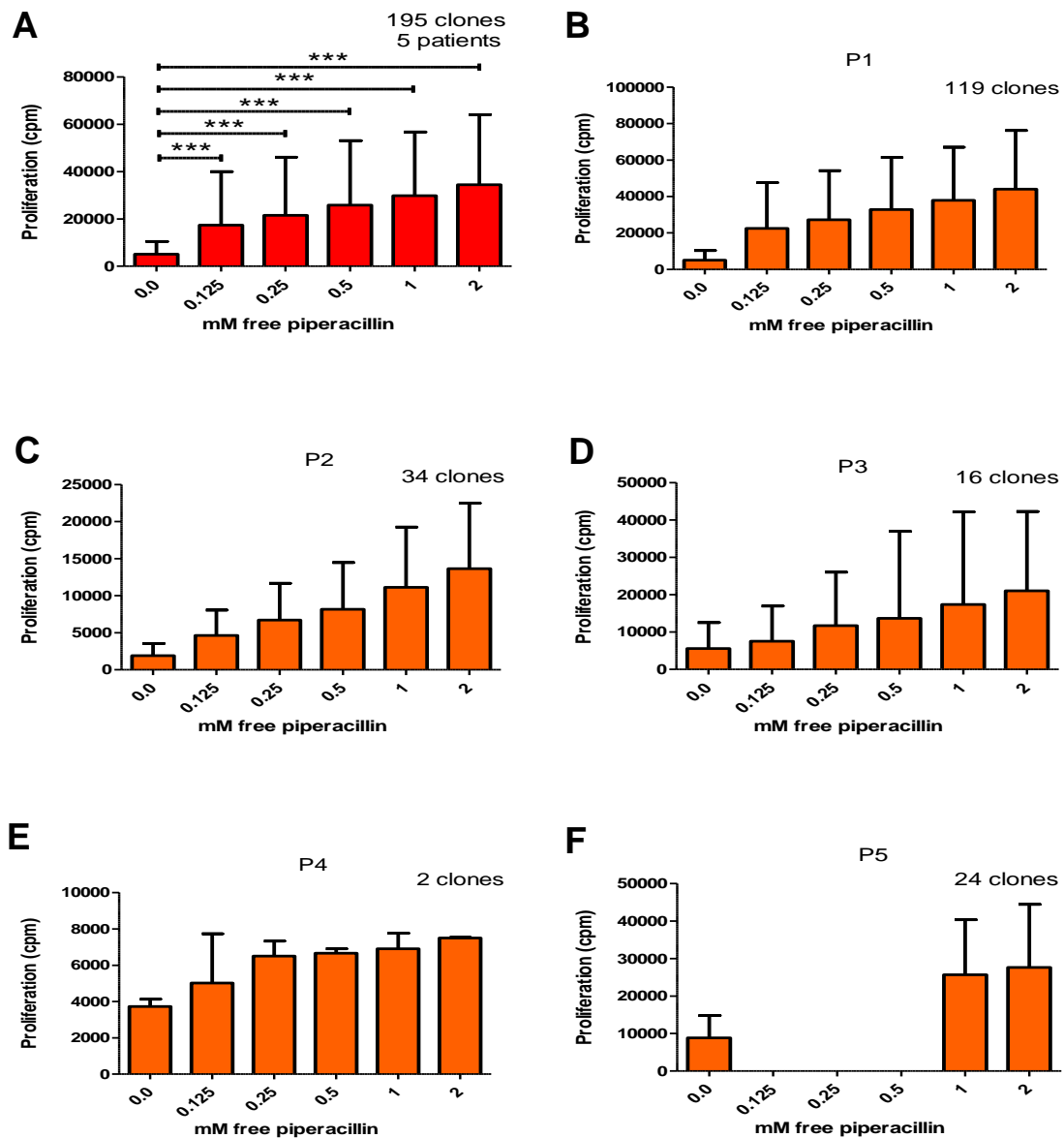
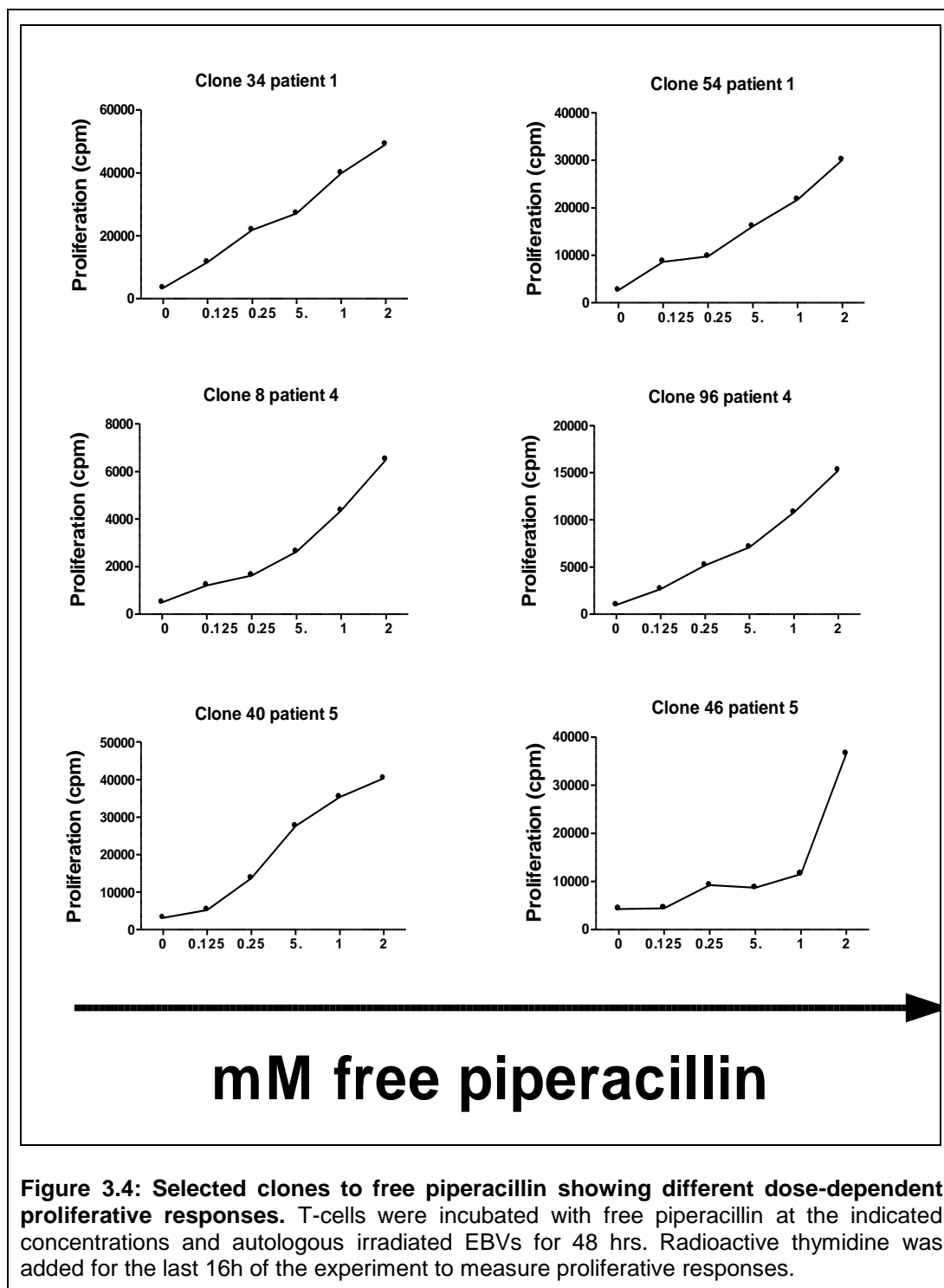


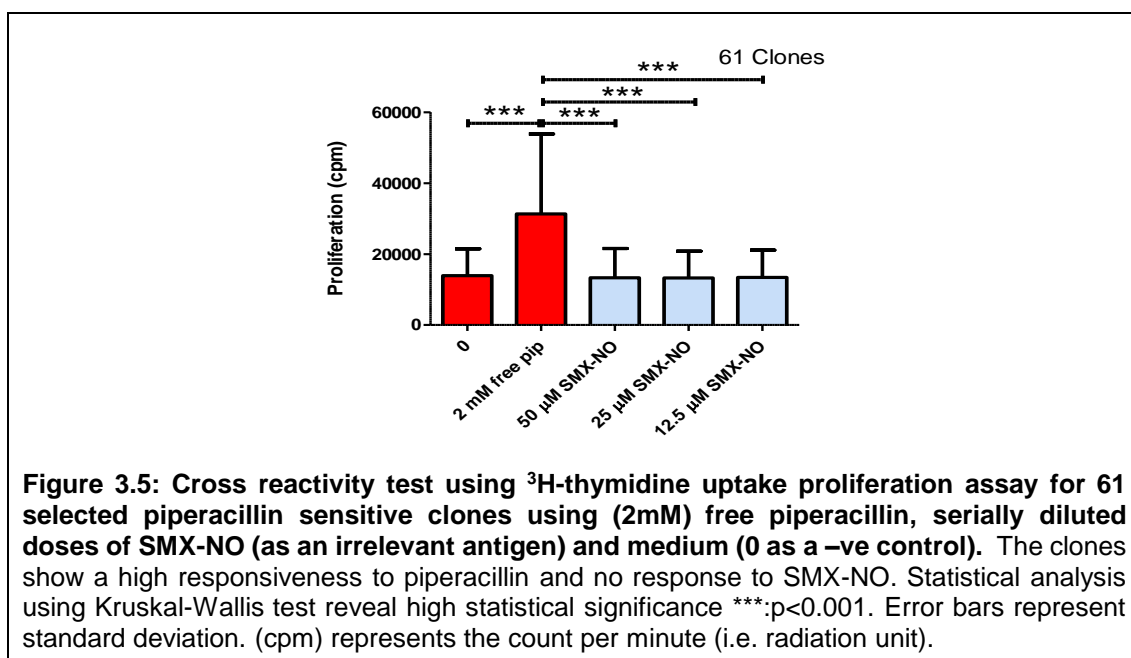
Figure 3.3: (A) ^3H -thymidine uptake assay for 195 selected piperacillin clones from 5 patients (P1-P5). (B,C,D,E and F) show the T-cells clones proliferative response for each patient separately. T-cells were incubated with free piperacillin and autologous irradiated APCs for 48 hrs. At last 16 hrs radioactive thymidine was added and followed by harvesting. Proliferation was determined by ^3H -thymidine incorporation. The clones show a dose-dependent response curve.. Statistical analysis using Kruskal-Wallis test reveal high statistical significance ***: $p < 0.001$. Error bars represent standard deviation. (cpm) represents the count per minute (i.e. radiation unit).

3.4.1.3 Reactivity of piperacillin-responsive clones with an unrelated drug antigen nitroso sulfamethoxazole

61 piperacillin-reactive clones from 5 patients were assessed for cross reactivity between free piperacillin and the unrelated drug metabolite antigen SMX-NO using ^3H thymidine-uptake to measure proliferation, T-cells were challenged with piperacillin (2mM) and 3 concentrations of SMX-NO (50, 25 and 12.5 μM ; shown previously to be optimal for the activation of SMX-NO-responsive clones) (Castrejon et al., 2010) in addition to the medium (-ve control).

The maximal response was for piperacillin with high statistical significance $p < 0.001$. Comparatively, significant proliferative responses were not observed with SMX-NO (**Figure 3.5**).



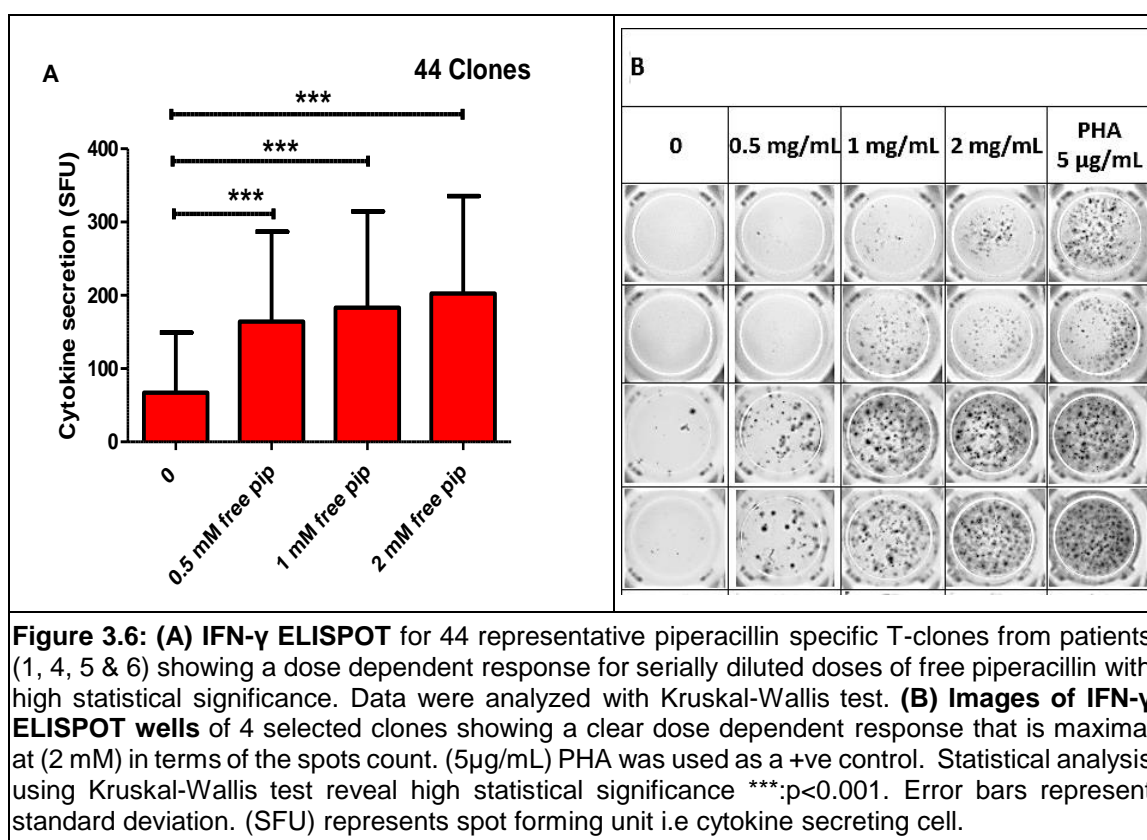


3.4.1.4 Measurement of cytokine secretion from the piperacillin-responsive T-cell clones

The reactivity of the clones which are sensitive and specific where assessed in terms of their cytokine expression using ELISPOT.

3.4.1.4.1 IFN- γ secretion

44 selected clones from patients 1, 3, 4 & 5 were analyzed for piperacillin mediated IFN- γ expression using serially diluted doses of free piperacillin starting with (2mM). PHA (5 $\mu\text{g}/\text{mL}$) was used as a +ve control and medium (0) as a -ve control. **Figure 3.6 (A)** shows the piperacillin dose-dependent release of IFN- γ from the clones. The maximal response was observed with 2mM free piperacillin with high statistical significance at p<0.001. In total, 86% of clones (n=38) were positive for IFN- γ secretion with an SI \geq 2. Images of representative clones with a dose-dependent proliferative response pattern are shown in **Figure 3.6 (B)**.

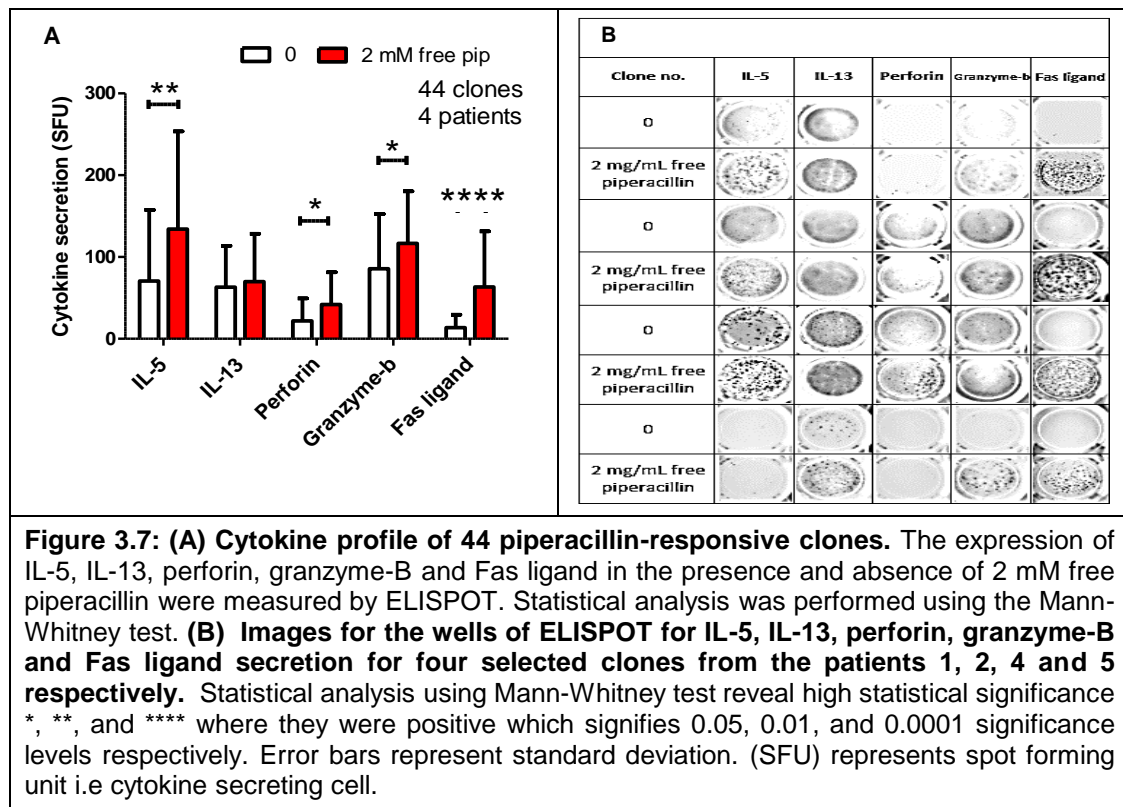


3.4.1.4.2 Interleukin-5, interleukin-13, perforin, granzyme-B and Fas ligand secretion

The profile of cytokines secreted from 44 piperacillin-responsive clones (the same clones selected for IFN- γ secretion) is shown in **Figure 3.7 (A)**. Twenty-two (50%) of piperacillin-reactive clones secreted IL-5, 17 (38.6%) secreted IL-13, 22 (50%) secreted perforin, 12 (27%) secreted granzyme-B and 23 (52%) secreted Fas ligand (SI ≥ 2). In terms of statistical significance there was a high statistical significance at *:p<0.05, *:p<0.01 and *** p<0.001 according to the Mann-Whitney test. Images for the wells of ELISPOT for IL-5, IL-13, perforin, granzyme-B and Fas ligand secretion for four selected clones from the patients are shown in **Figure 3.7 (B)**.

In terms of the statistical significance of difference between the control and the free piperacillin the results were as follows in descending order: For Fas ligand

($p < 0.0001$), for IL-5 ($p = 0.0095$), less with perforin ($p = 0.014$) and granzyme-B ($p = 0.027$), and non-statistically significant for IL-13 ($p = 0.63$) as analyzed by Mann –Whitney test.



3.4.1.5 Measurement of the T-cell receptors and chemokine receptors expressed on piperacillin-responsive clones

3.4.1.5.1 CD4/CD8 receptor expression:

79 reactive clones from the patients (1-5) were analyzed by flow cytometry. This has revealed that the majority of clones are CD4+ ($n = 73$, 92%), while CD8+ clones account for only 5% ($n = 4$). Finally, only 2 double positive CD4+CD8+ clones were observed. All of the clones displayed a memory T-cells phenotype ($n = 97$, 100%) expressing high levels of CD45RO. A detailed description of the phenotype of the clones is listed in **Table 3.3**.

Images for the selected piperacillin clones showing gating and the individual populations are shown in . The T-cell population was gated according

Table 3.3: The number of piperacillin-specific clones analyzed from each patient and their phenotypic subdivisions.

Patient ID number	Number of reactive specific clones	Number of CD4+ clones	Percentage of CD4+ clones	Number of CD8+ clones	Percentage of CD8+ clones	Number of CD4+CD8+	Percentage of CD4+CD8+ clones
P 1	42	37	88.10%	3	7.14%	0	0.00%
P 2	7	6	85.71%	0	0.00%	1	14.29%
P 3	28	26	92.86%	1	3.57%	1	3.57%
P 4	2	2	100.00%	0	0.00%	0	0.00%
P 5	2	2	100.00%	0	0.00%	0	0.00%
Total	81	73	90.12%	4	4.94%	2	2.47%

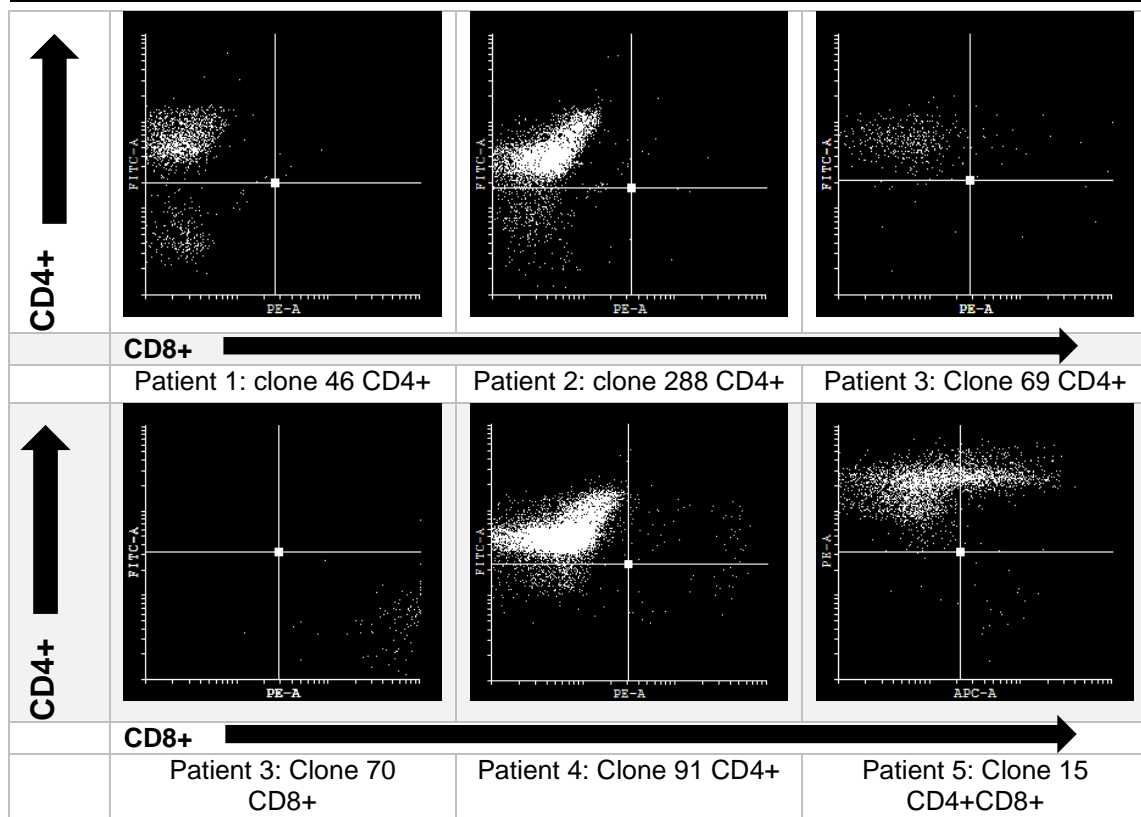
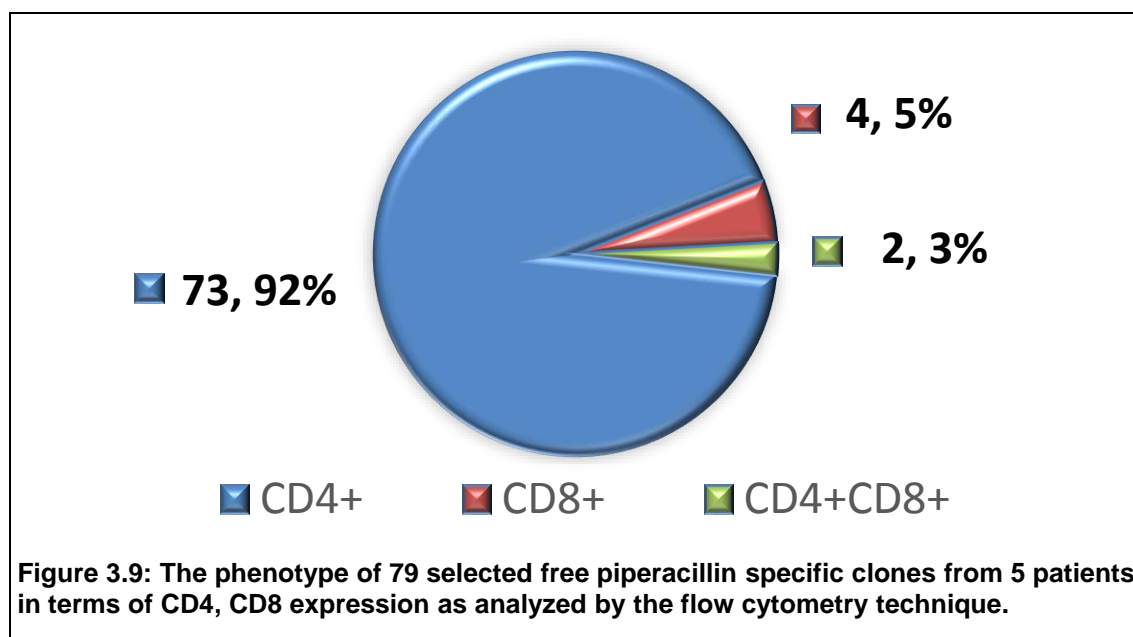


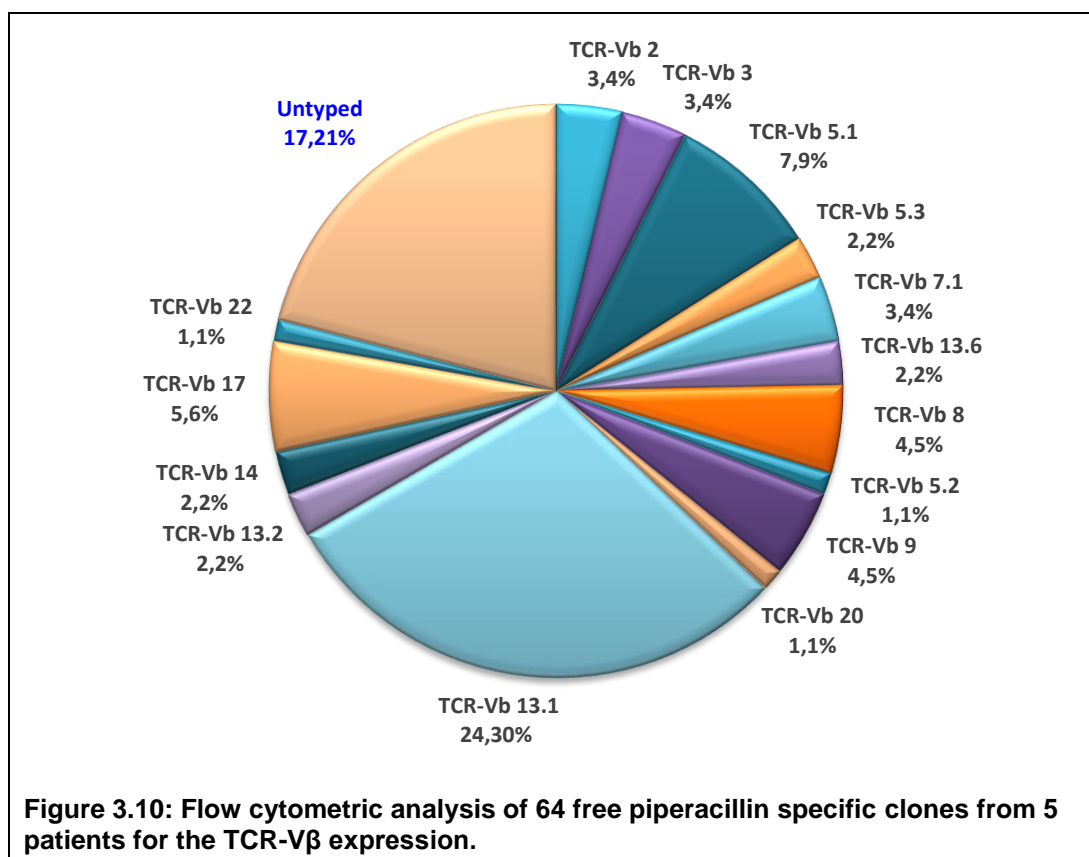
Figure 3.8: Images of the flow cytometry data gating on the FACS machine showing the phenotype of selected piperacillin specific clones in terms of CD4/CD8 expression. Cells are plotted on a dot plot and gated according to the staining of the fluorochrome labelled antibodies (CD4+ for FITC and CD8+ for PE) into four quadrants. So, when a clone shows a dots signal at the FITC quadrant only (upper left) this denoted that it is CD4+ clone, while if the signal at PE quadrant alone (lower right), this denotes that it is a CD8+. If dots are present in upper right quadrant, it denotes that the clones is CD4+CD8+ (double positive).

to the labelling with CD4+ for FITC and CD8+ for PE. When a clone displayed a signal in the FITC quadrant only (upper left) (clones 46, 288, 69 and 91) this denoted that they are CD4+ clones, while if the signal was in the PE quadrant alone (lower right), this denotes that it is a CD8+ (clone 70). If a signal is present in upper right quadrant it denotes that the clone is CD4+CD8+ (double positive) (clone 15).

3.4.1.5.2 Characterisation of TCR-V β expression on the T-cell clones from hypersensitive patients

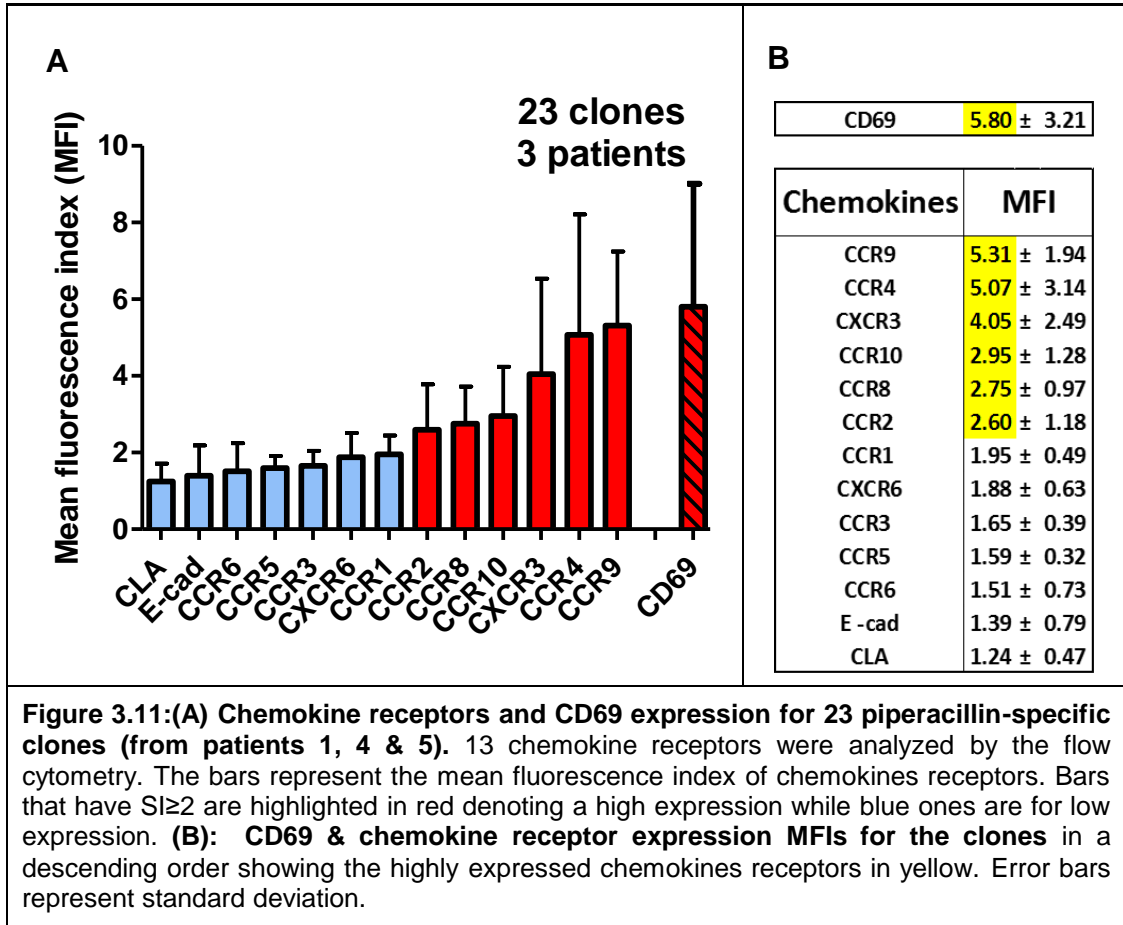
After characterizing the clones in terms of their CD4/CD8 expression, we carried out analysis of TCR-V β receptors. 64 clones from the patients (1-5) were analyzed by flow cytometry. 24 clones were found to express high levels of a single TCR-V β 13.1 (30%). However, an array of TCR-V β s were expressed on the other clones. As shown in **Figure 3.10** 15 TCR-V β s were detected on the piperacillin-responsive clones with variable expression.





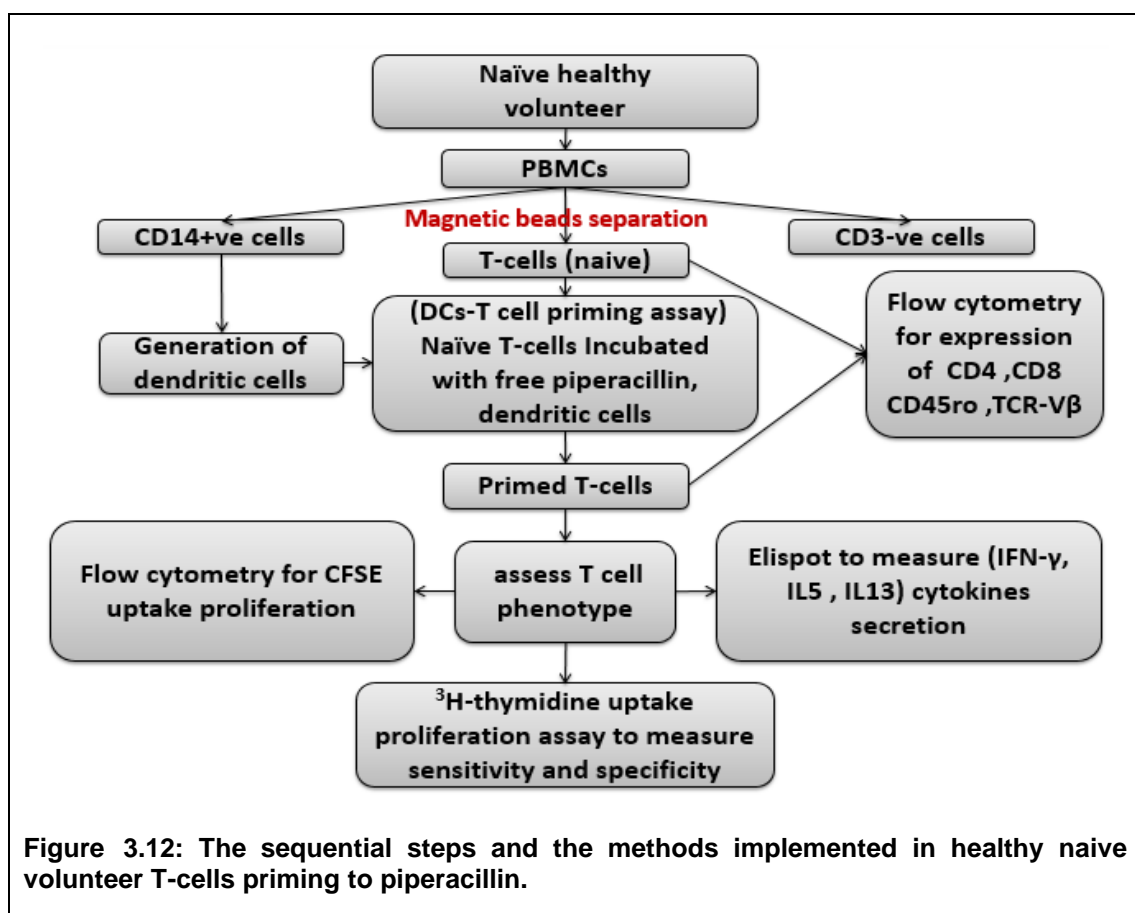
3.4.1.6 Characterization of chemokine receptors and CD69 expression on piperacillin-responsive T-cell clones from hypersensitive patients

The chemokine receptors and CD69 expression on 23 clones from patients 1, 2 and 4 were analyzed by flow cytometry. **Figure 3.11** shows that clones expressed for CD69 (5.80 ± 3.21 ; mean fluorescence index), CCR4 (5.07 ± 3.13), CCR9 (5.31 ± 1.93), CXCR3 (4.04 ± 2.48), CCR10 (2.95 ± 1.28), CCR2 (2.59 ± 1.18) and CCR8 (2.75 ± 0.96).



3.4.2 Healthy naive volunteer T-cell priming to piperacillin

Naïve T-cells from healthy piperacillin-naïve volunteers were primed and tested for sensitivity, specificity, phenotype and cytokines secretion as shown in **Figure 3.12**.



Three healthy piperacillin naïve volunteers were selected for T-cell priming experiments. Blood samples were collected and the PBMCs were separated. Then, DC-T-cell priming was performed to activate the naïve T-cells to free piperacillin. Thus, drug-responsive T-cell lines were generated. **Table 3.4** shows the basic characteristics of the donors.

Table 3.4: The demographics of the three normal healthy volunteers enrolled in this study.				
Donor ID	Age	Sex	History of allergies	Medical history
HVN-051	40	Male	Nil	Not relevant
HVN-066	36	Male	Nil	Not relevant
HVN-089	37	Male	Nil	Not relevant

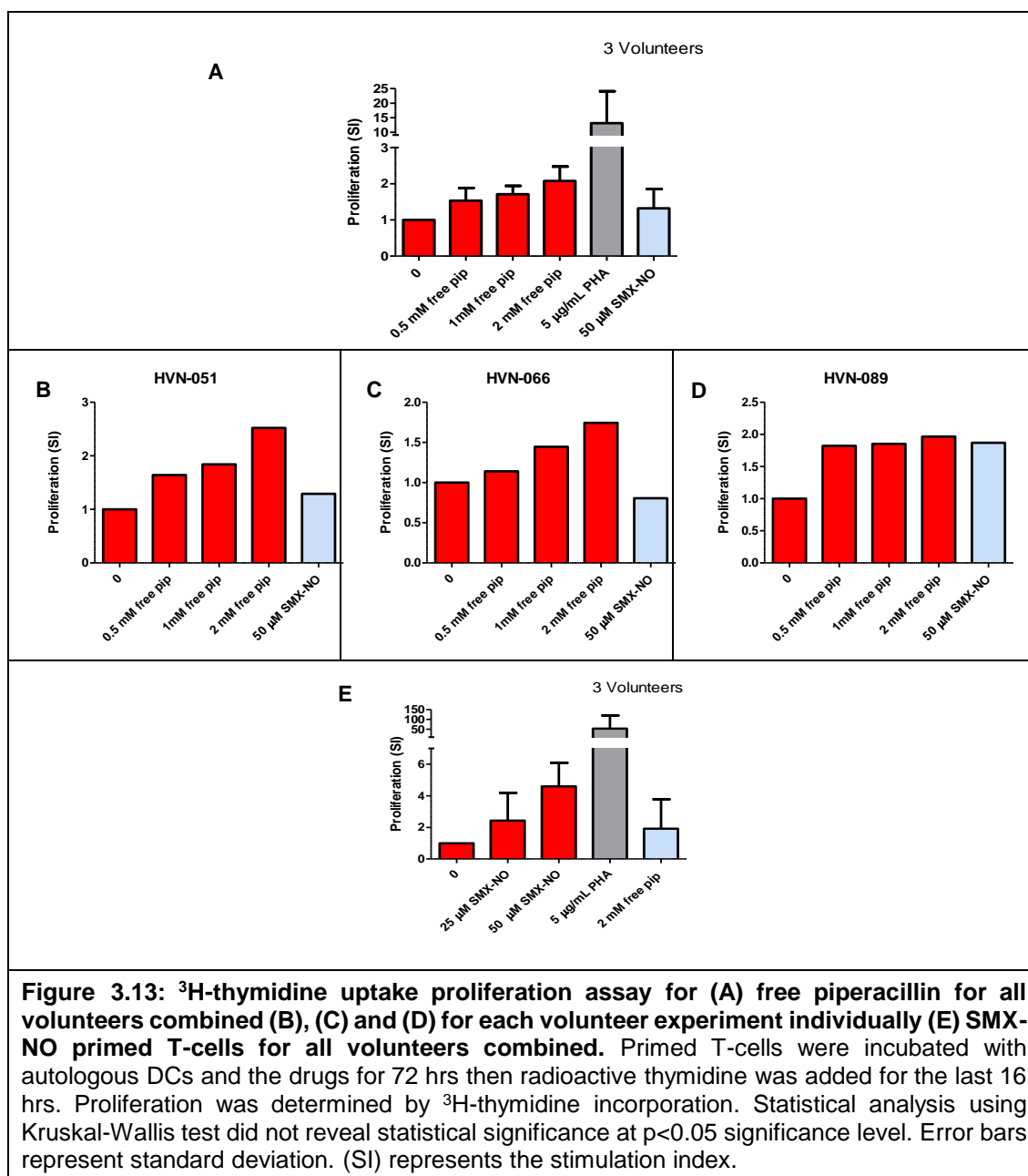
3.4.2.1 T-cell reactivity and specificity assays

3.4.2.1.1 ³H-thymidine uptake proliferation assay

On day 15 of the T-cell priming assay, T-cells were harvested from the co-culture plates and then incubated with autologous DCs and the test compounds (serially diluted doses of free piperacillin, 5 µg/mL PHA (positive control) and 50 µM SMX-NO (irrelevant drug antigen).

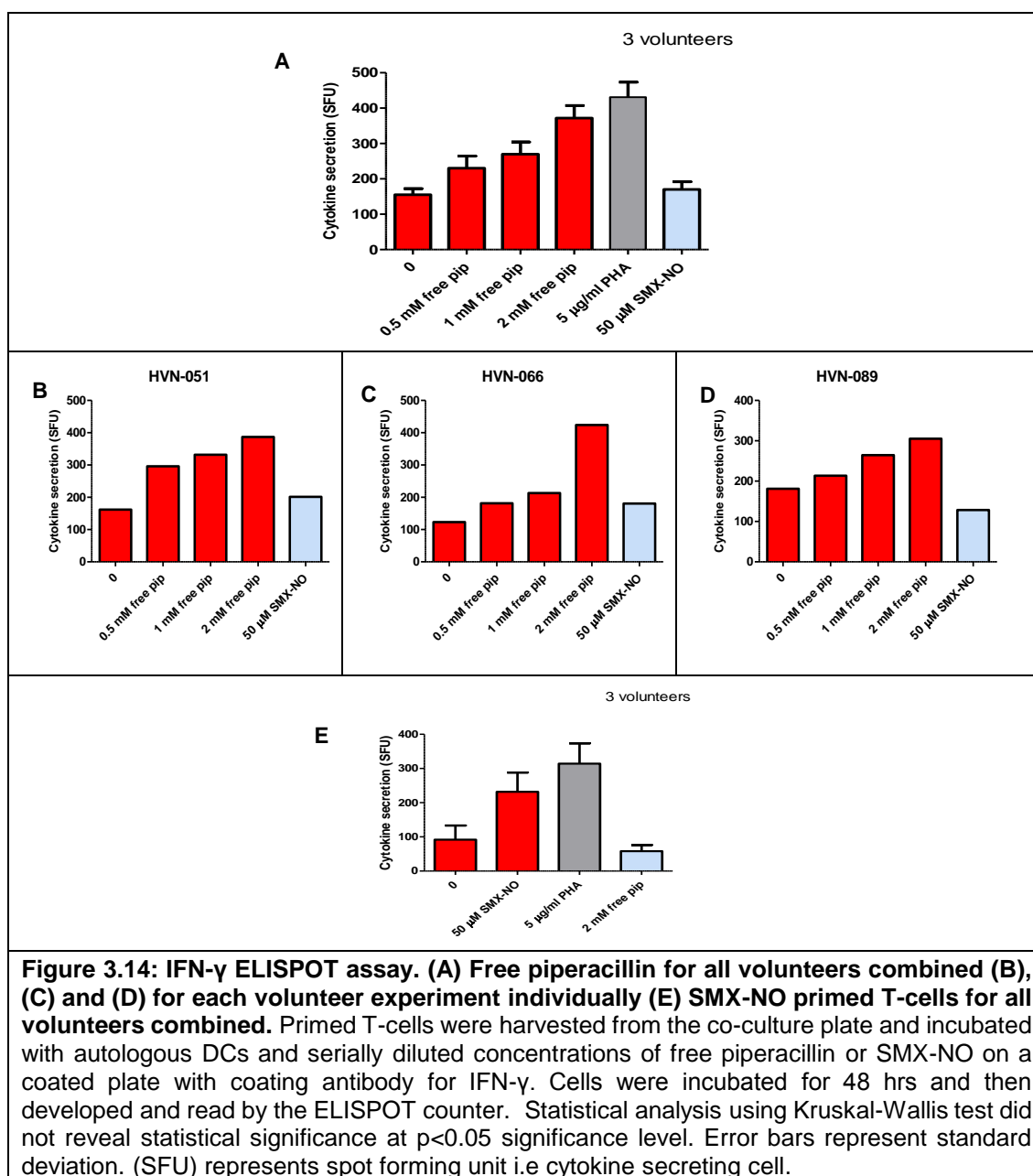
A dose-dependent increase in T-cell proliferation was observed when the primed T-cells were restimulated with piperacillin (**Figure 3.13 A**). In contrast, the T-cells were not activated with SMX-NO. The results of individual experiments on each volunteer are illustrated in **Figure 3.13 B, C and D** showing reactivity to free piperacillin.

In similar experiments, naïve T-cells were primed to SMX-NO. An increase in proliferation was observed when the primed T-cells were restimulated with SMX-NO, but not piperacillin **Figure 3.13 E**.



3.4.2.1.2 ELISPOTS

On day 15 of the T-cell priming assay, the primed T-cells were tested for cytokine release to serially diluted doses of free piperacillin, PHA (5 µg/mL; as a +ve control) and SMX-NO (50 µM; as an irrelevant drug antigen).




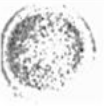
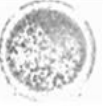

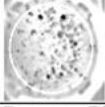
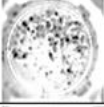
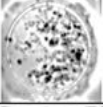
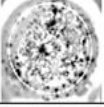
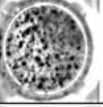
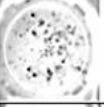
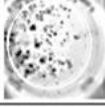

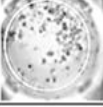
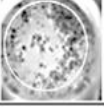
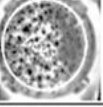
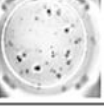

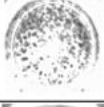
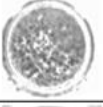

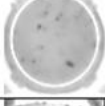
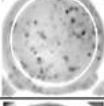
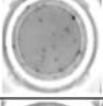
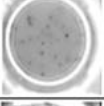
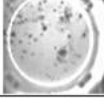
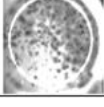

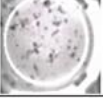


• IFN-γ ELISPOT

Figure 3.14 A: shows that the piperacillin primed T-cells secreted IFN-γ in a concentration-dependent fashion when restimulated with the drug. Similar to the patients cells, maximal responses were observed with 2mM piperacillin (372 ± 60.9 SFU). The piperacillin-primed T-cells were not activated with SMX-NO

(170.2 ± 37.58 SFU). Results of individual experiments for each volunteer cells are illustrated in **Figure 3.14 B, C and D**.

Table 3.5: IFN- γ ELISPOT for free piperacillin and SMX-NO. Primed T-cells were harvested from the coculture plate and incubated with autologous DCs and serially diluted doses of free piperacillin and SMX-NO on a coated plate with the coating antibodies for IFN- γ and incubated for 48 hrs and then developed and read by the ELISPOT counter. The table below shows images of wells of the ELISPOT plate.

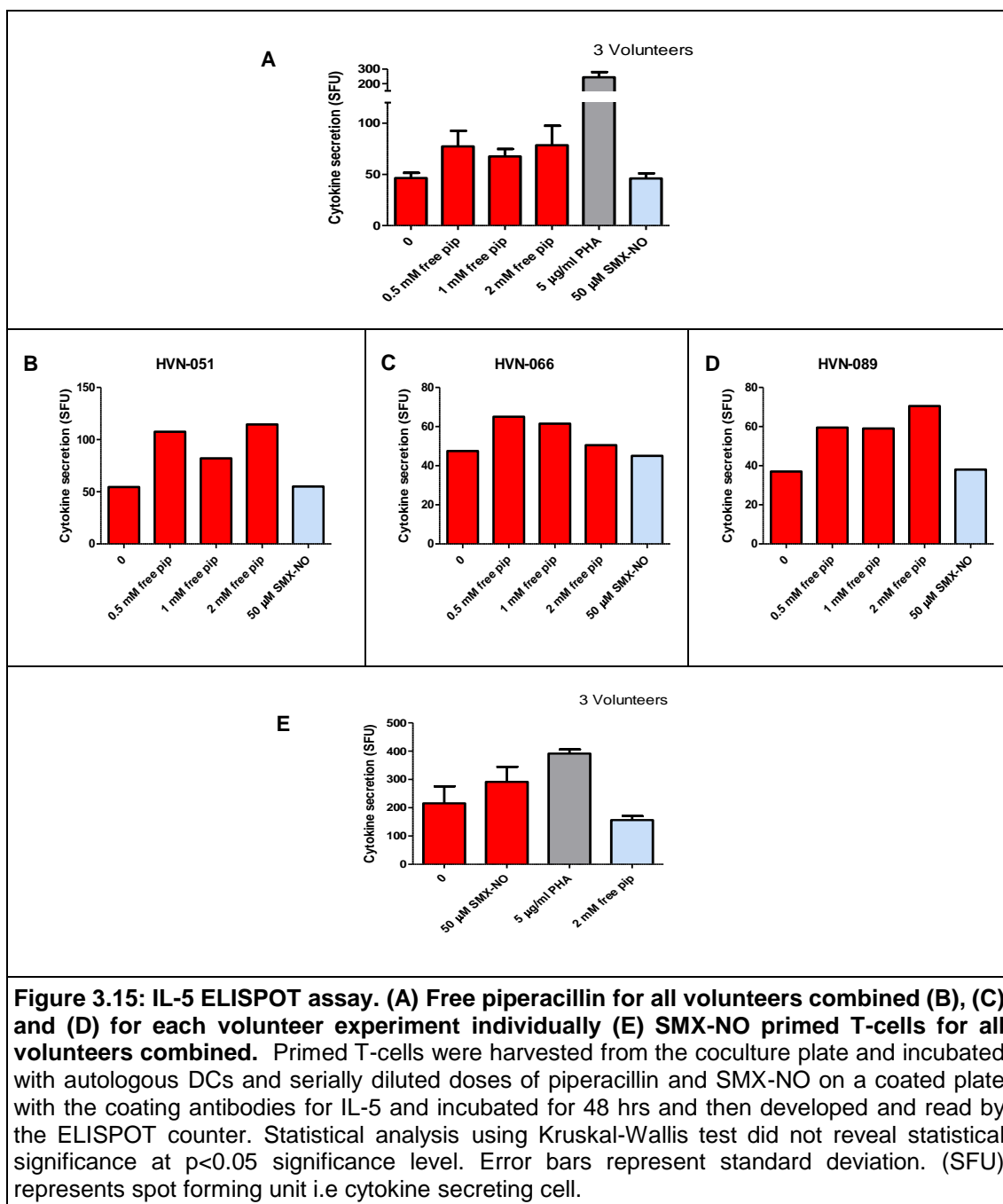
	Volunteer ID.	0	0.5 mM free pip	1 mM free pip	2 mM free pip	5 mg/ml PHA	50 μ M SMX-NO
Free piperacillin primed T-cells	051						
	066						
	089						
		0	50 μ M SMX-NO	5 μ g/ml PHA	2 mM free pip		
SMX-NO primed T-cells	051						
	066						
	089						

Similar experiments were conducted with SMX-NO-primed T-cells. T-cells secreted IFN- γ in the presence of SMX-NO, but not piperacillin (**Figure 3.14 E**).

Table 3.5 shows images of the IFN- γ ELISPOT where it can be clearly seen that piperacillin-primed T-cells were activated with piperacillin, but not SMX-NO, whereas the SMX-NO primed cells were activated with SMX-NO, but not piperacillin.

IL-5 ELISPOT

Piperacillin primed T-cells secreted low levels of IL-5. (2mM free piperacillin;



8.5±32.74 SFU) with no cross reactivity to SMX-NO (46±8.54 SFU) (**Figure 3.15**

A). In contrast, SMX-NO primed T-cells secreted significantly higher levels of IL-5 (50 μ M SMX-NO; 215.5 ± 104.4 SFU), but again no cross reactivity with piperacillin was observed (**Figure 3.15 B**). Images of the ELISPOT are shown in **Table 3.6**.

Table 3.6. IL-5 ELISPOT images for free piperacillin and SMX-NO conditions. Primed T-cells were harvested from the coculture plate and incubated with autologous DCs and serially diluted doses of piperacillin and SMX-NO on a coated plate with the coating antibodies for IL-5 and incubated for 48 hrs and then developed and read by the ELISPOT counter.

Free piperacillin primed T-cells						
Volunteer ID.	0	0.5 mM free pip	1 mM free pip	2 mM free pip	5 μ g/ml PHA	50 μ M SMX-NO
051						
066						
089						
SMX-NO primed T-cells						
	0	50 μ M SMX-NO	5 μ g/ml PHA	2 mM free pip		
051						
066						
089						

• IL-13 ELISPOT

The free piperacillin primed T-cells secreted IL-13 in a dose-dependent manner when restimulated with piperacillin. SMX-NO primed T-cells secreted IL-13 response to SMX-NO, but not piperacillin (**Figure 3.18; Table 3.6**).

Moreover, these results for IL-13 ELISPOT proves that there is no cross reactivity between free piperacillin and SMX-NO.

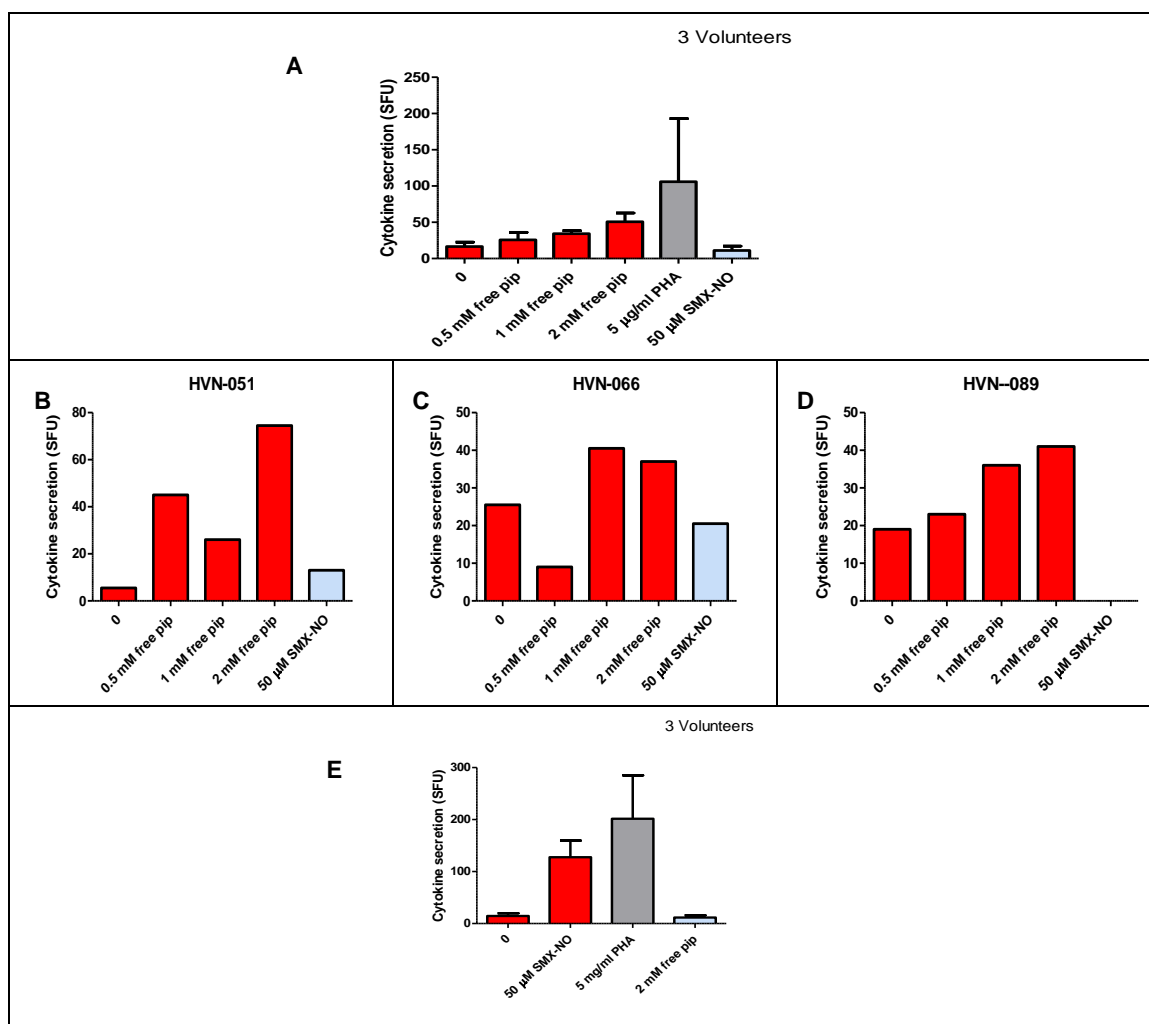


Figure 3.16: IL-13 ELISPOT. (A) Free piperacillin for all volunteers combined (B), (C) and (D) for each volunteer experiment individually (E) SMX-NO primed T-cells for all volunteers combined. Primed T-cells were harvested from the coculture plate and incubated with autologous DCs and serially diluted doses of piperacillin and SMX-NO on a coated plate with the coating antibodies for IL-13 and incubated for 48 hrs and then developed and read by the ELISPOT counter. Statistical analysis using Kruskal-Wallis test did not reveal statistical significance at $p < 0.05$ significance level. Error bars represent standard deviation. (SFU) represents spot forming unit i.e cytokine secreting cell.

3.4.2.2 Flow cytometric analysis of T-cell phenotype and TCR-V β expression pre- and post-priming

Naïve T-cells were analyzed using flow cytometry (on day 7; just prior to priming) and then the analysis was repeated after priming (on day 18). The results were compared to determine the ratio of CD4 & CD8 receptor expression, priming

Table 3.7: IL-13 ELISPOT images for the free piperacillin and the SMX-NO primed T-cells. Primed T-cells were harvested from the coculture plate and incubated with autologous DCs and serially diluted doses of piperacillin and SMX-NO on a coated plate with the coating antibodies for IL-13 and incubated for 48 hrs and then developed and read by the ELISPOT counter.

	Volunteer ID.	0	0.5 mM free pip	1 mM free pip	2 mM free pip	5 μ g/ml PHA	50 μ M SMX-NO
Free piperacillin primed T-cells	051						
	066						
	089						
		0	50 μ M SMX-NO	5 μ g/ml PHA	2 mM free pip		
SMX-NO primed T-cells	051						
	066						
	089						

Table 3.8: The average percentages of the T-cell phenotypes in prepriming (day 0) and post priming (day 18) flow cytometry analysis.

Cells phenotype	Pre-priming average percentage	Post-priming average percentage	Change ratio
CD4+ cells	50.03%	40.66%	0.81
CD8+ cells	46.43%	35.11%	0.75
CD4+CD8+ cells	0.84%	7.91%	9.41
CD4-CD8- cells	2.69%	16.29%	6.04

Table 3.9. Percentages of the T-cells pre and post priming in terms of their CD4, CD8 receptors expression as analyzed by the flow cytometry technique. The analysis was conducted on day 7 for the naïve T-cells (prepriming) and on day 18 for the primed T-cells (post priming).

	HVN-051		HVN-066		HVN-089	
	Pre-priming	Post-priming	Pre-priming	Post-priming	Pre-priming	Post-priming
CD4+ cells	41.9%	63.5%	43.3%	29.7%	64.9%	28.8%
CD8+ cells	55.7%	29.2%	51.4%	47.0%	32.2%	29.2%
CD4+CD8+ cells	0.7%	3.7%	1.4%	16.8%	0.4%	3.2%
CD4-CD8- cells	1.7%	3.6%	3.9%	6.5%	2.5%	38.8%

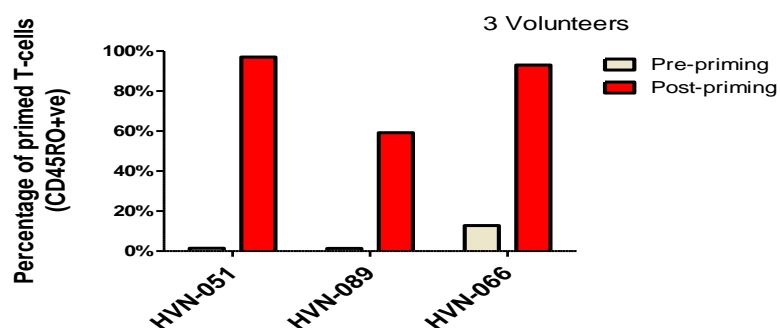
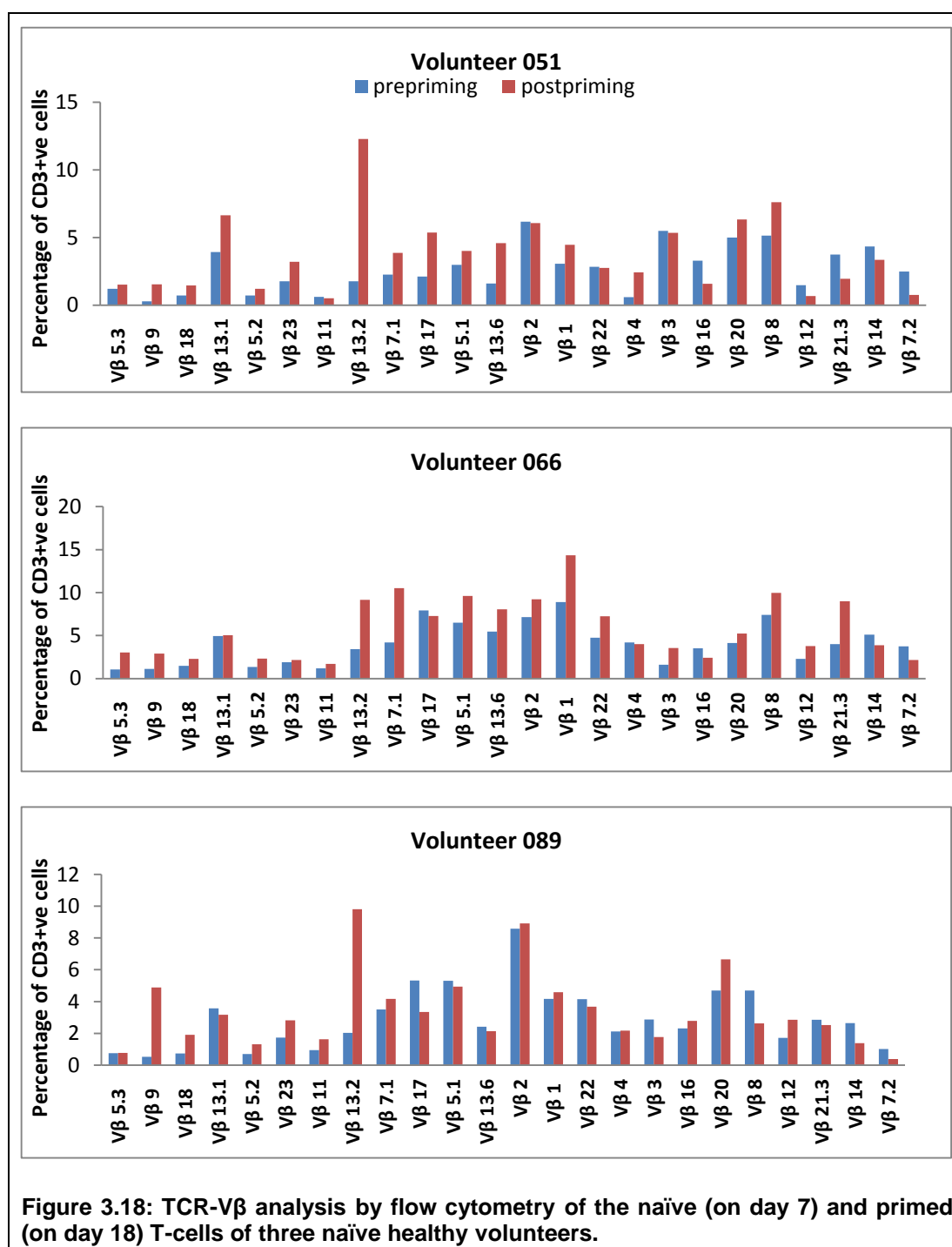


Figure 3.17: Percentages of the T-cells pre and post priming in terms of their CD45RO receptor expression as analyzed by the flow cytometry technique. The analysis was conducted on day 7 for the naïve T-cells (prepriming) and on day 18 for the primed T-cells (post priming).

magnitude (in terms of CD45RO expression) and the skewing of TCR-V β expression.

The ratio of CD4+ and CD8+ T-cells did not significantly change pre- and post-priming of the naive-primed T-cells. In contrast, an increase in the number of double positive (DP) CD4+CD8+ T-cells was seen (from 0.84% pre-priming to 7.91% post-priming). Moreover, the double negative (DN) CD4-CD8- cell number increased (from 2.69% pre-priming to 16.29% post-priming) (**Table 3.8**).

An increased expression of CD45RO was observed with the primed cells. CD45RO expression ranged from 1-13% on the pre-primed T-cells. In contrast, this increased to 60-97% on the T-cells post priming (**Figure 3.17**).



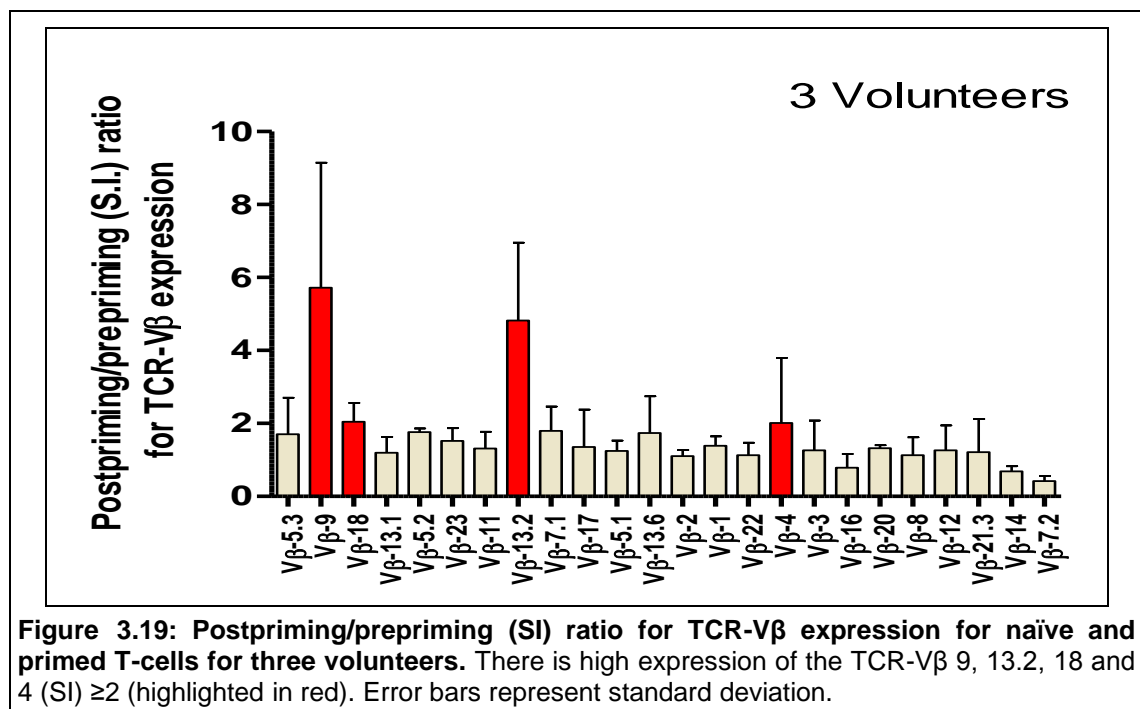
A heterogeneous change in TCR-Vβ expression was observed post-priming with piperacillin (**Figure 3.18**). Vβ9 (5.71 ± 3.42 ; ratio of postpriming/prepriming

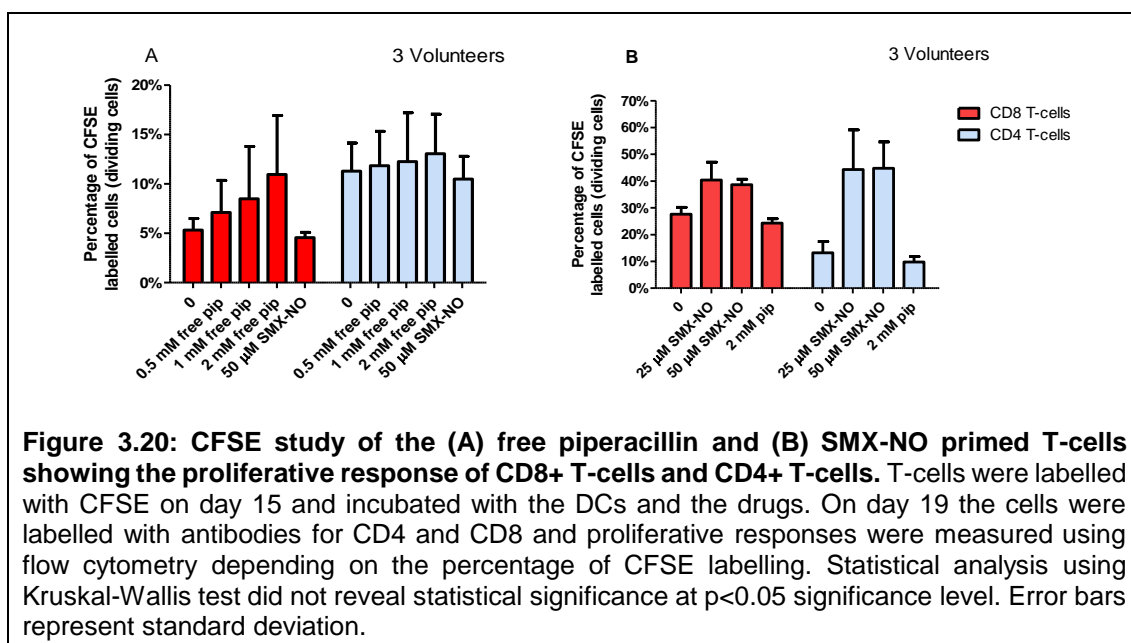
expression), V β 13.2 (2.04 ± 0.51), V β 18 (4.81 ± 2.13) and V β 4 (2.01 ± 1.77) showed a marked increase in V β expression in the post priming stage (i.e., postpriming/prepriming ≥ 2).

3.4.2.3 CFSE analysis

On day 15, free piperacillin and SMX-NO primed T-cells were labelled with CFSE, restimulated and then analyzed on day 19 by flow cytometry.

CD8+ T-cells from piperacillin-primed cultures showed a strong dose-dependent proliferative response when restimulated with piperacillin. In comparison, the CD4+ T-cells proliferated to a much lesser extent (**Figure 3.20 A**). The SMX-NO primed CD4+ and CD8+ T cells show a dose-dependent response when restimulated with SMX-NO (**Figure 3.20 B**).

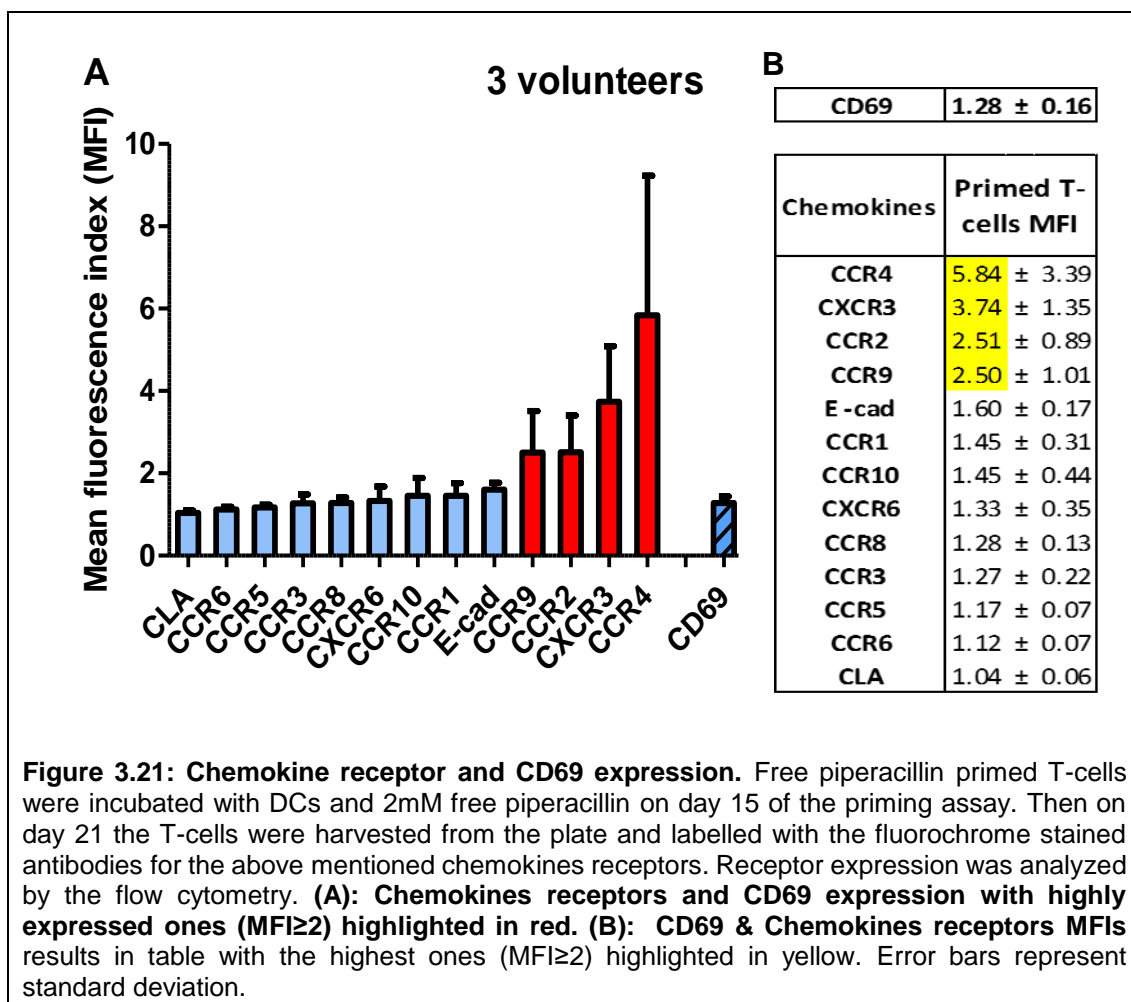




3.4.2.4 Chemokine receptor analysis

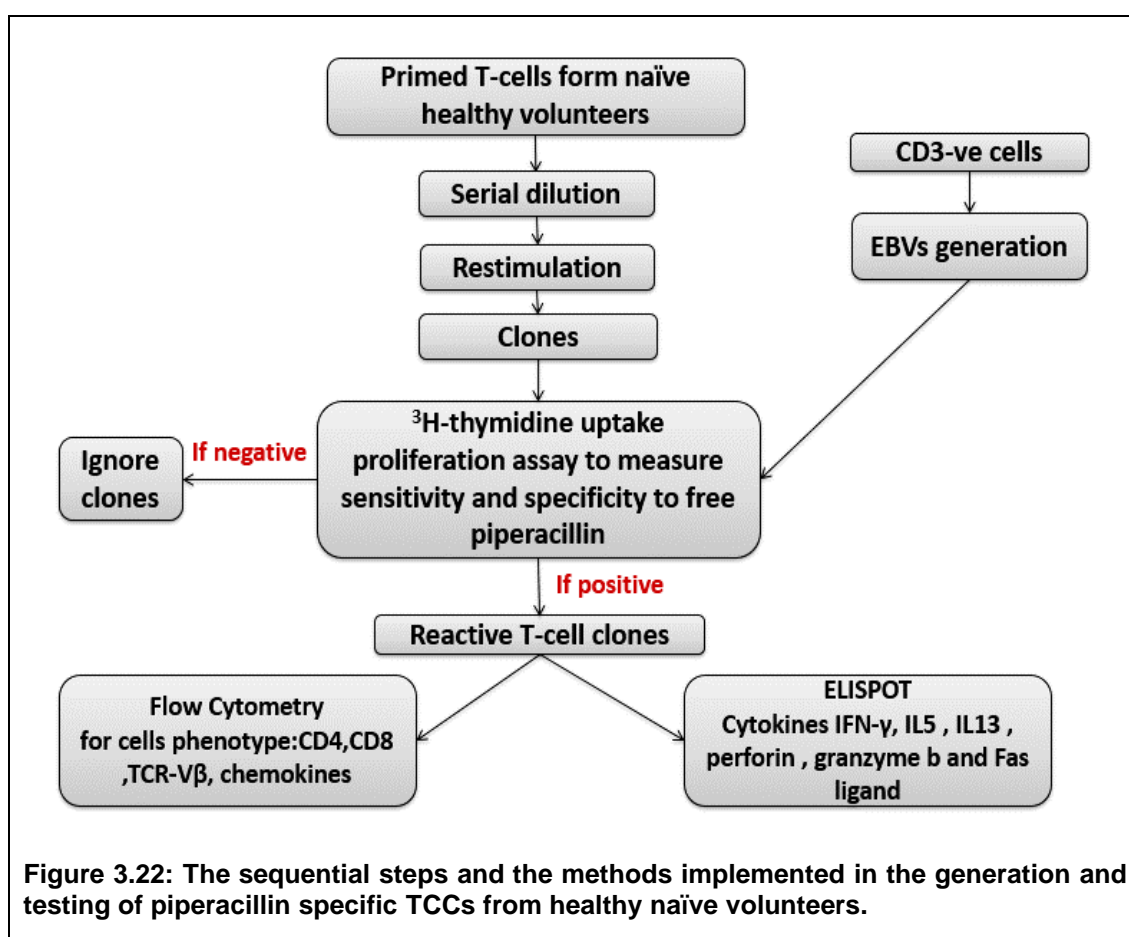
On day 15, free piperacillin primed T-cells were incubated with DCs and free piperacillin (2mM). Seven days later T-cells were harvested, labelled with a panel of chemokine receptor antibodies and then analyzed by flow cytometry.

An increased expression of CCR4 (5.84 ± 3.39), CXCR3 (3.74 ± 1.35), CCR2 (2.51 ± 0.89) and CCR9 (2.5 ± 1.01) was observed on T-cells when pre- and post-piperacillin restimulation cells were compared (**Figure 3.21**).



3.4.3 Generation of clones from piperacillin-primed T-cells from naïve healthy volunteers

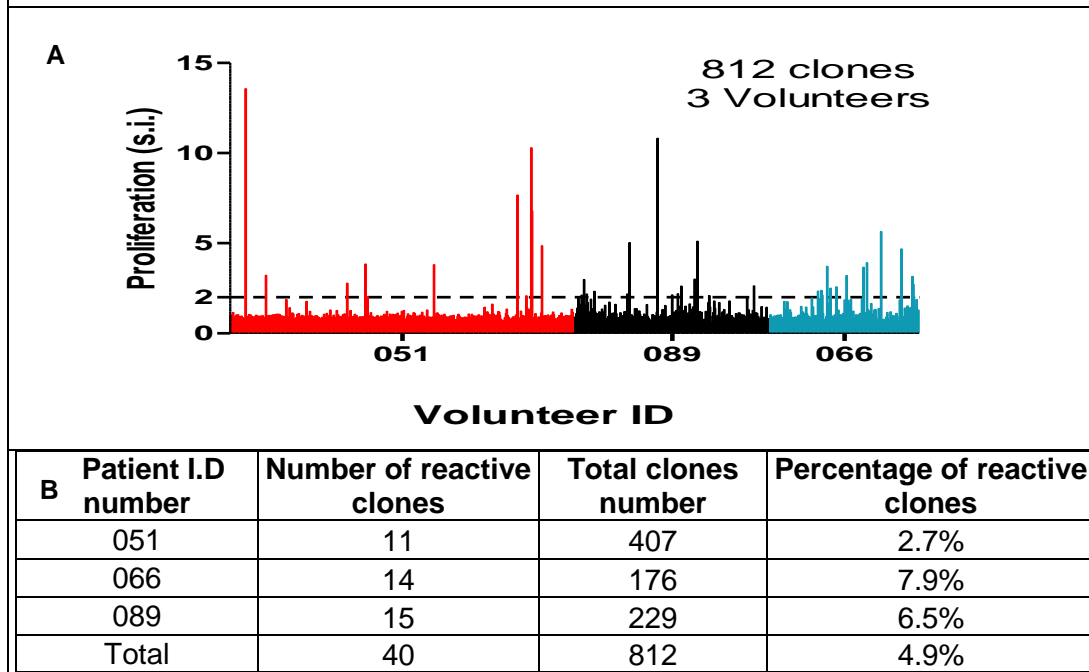
After the completion of the ELISPOT and ^3H -thymidine uptake assays that confirmed priming to free piperacillin was successful, clones were generated using serial dilution. In addition, autologous EBVs were generated according to the scheme shown in **Figure 3.22** and used as a source of autologous antigen presenting cells.



Well-growing clones were tested for piperacillin reactivity using a lymphocyte proliferation assay and if responsive ($\text{SI} \geq 2$), they were expanded via mitogen stimulation for more detailed phenotypic and functional analysis.

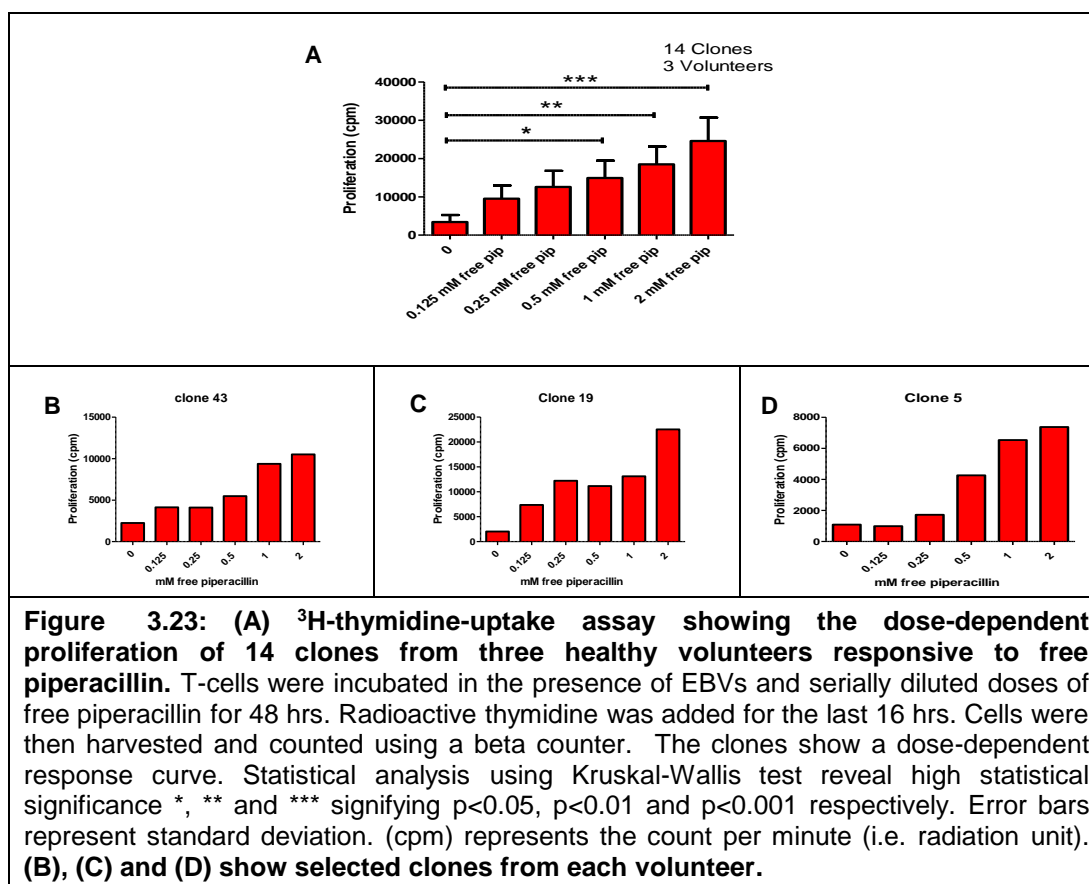
In total, 40 piperacillin-responsive clones (out of 812) were detected (**Table 3.10**).

Table 3.10: Clones generated from piperacillin-primed T-cells from three healthy volunteers. 812 clones were generated by the technique of serial dilution. Autologous EBVs were generated and used as antigen presenting cells. The clones were tested with vehicle control and 2 mM free piperacillin. If SI ≥ 2 the clone was regarded as sensitive. **(A):** Figure shows SI of all clones generated. **(B)** Table shows the numbers and percentages of sensitive clones from each volunteer.



3.4.3.1 Quantitative assessment of clones reactivity to piperacillin using ^3H - thymidine uptake assay

14 clones showing initial responses to free piperacillin were selected and tested using ^3H -thymidine-uptake to explore dose-dependent proliferative responses. All clones proliferated in a dose-dependent manner, with the strongest response detected with 2mM piperacillin. Since several clones proliferated only weakly following piperacillin treatment, a SI threshold of 1.5 was accepted from this assay and onwards as a drug-specific effect (**Figure 3.23**). Statistical significance was observed with all clones when medium control was compared with 2 or 1 mM free piperacillin at **: <0.01, * = <0.05 respectively as analyzed by Kruskal-Wallis test. **Figure 3.25 (B), (C) and (D)** show selected clones from each volunteer with their dose response curve.

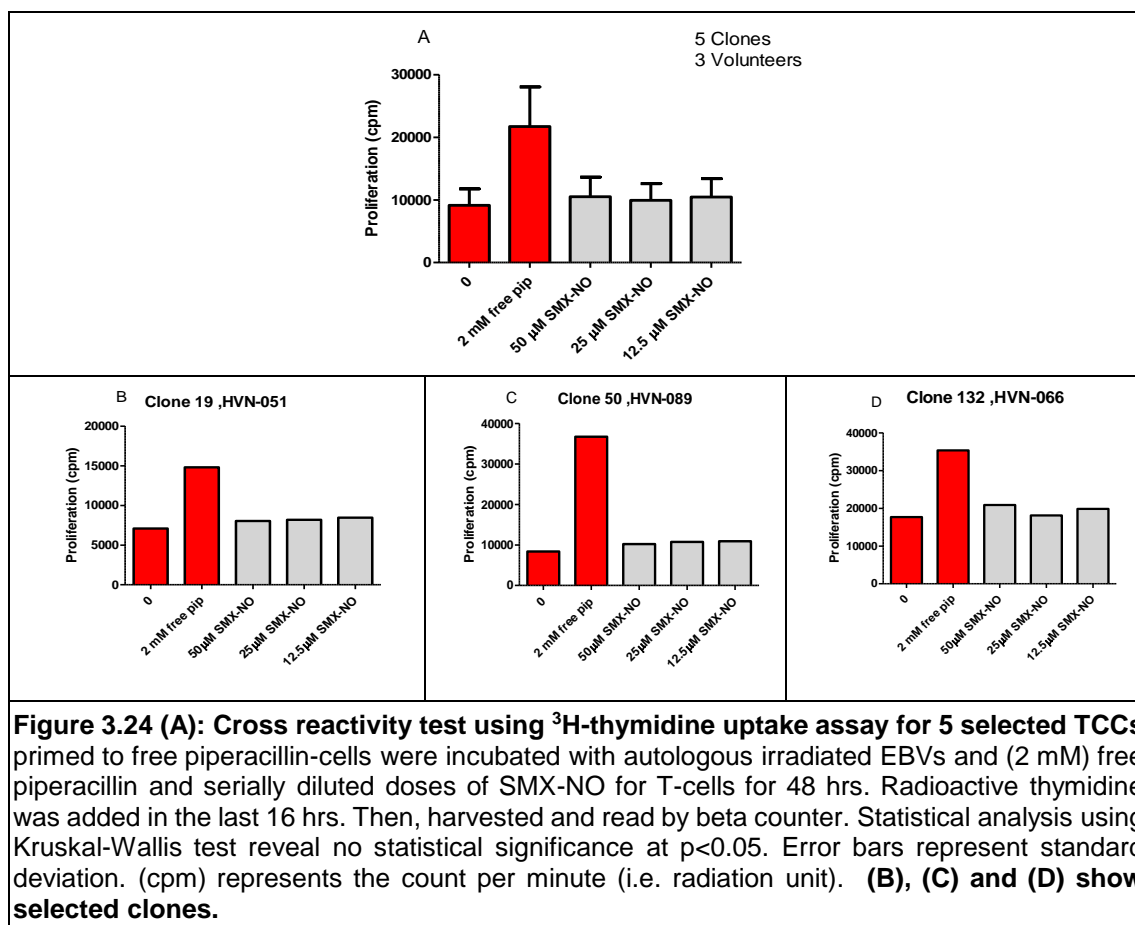


3.4.3.2 Reactivity of the piperacillin clones against nitroso sulfamethoxazole using ^3H -thymidine uptake assay

Five clones were selected to test for reactivity against the irrelevant drug antigen SMX-NO. A strong proliferative response to free piperacillin (24597.4 ± 22768.9 cpm; 2 mM) was observed. In contrast, clones were not activated with SMX-NO. **Figure 3.24 (A).** **Figure 3.24 (B), (C) and (D)** show proliferative responses of representative clones.

3.4.4 Quantitative and Qualitative measurement of cytokine secretion profile of the T-cell clones by ELISPOT

14 piperacillin-specific clones were tested for the cytokines they secrete after drug stimulation (IFN- γ , IL-5, IL-13, perforin, granzyme-B and Fas ligand) using ELISPOT.



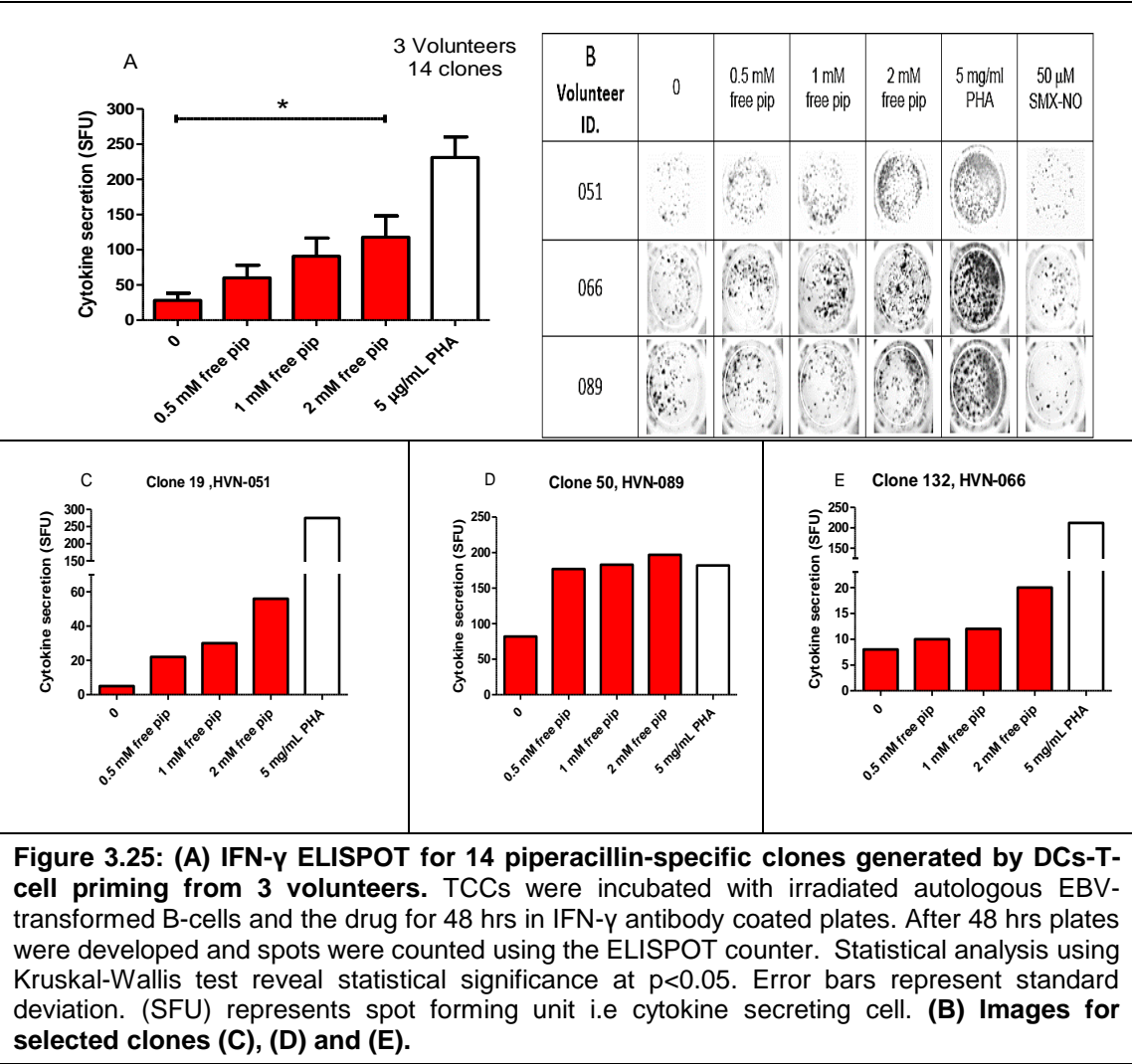
- **IFN- γ secretion**

A dose-dependent increase in IFN- γ secretion was observed when clones were activated with piperacillin. The maximal response was seen with 2mM free piperacillin (117.7 ± 113.6 cpm) with a statistical significance at $^*: p < 0.05$ as analyzed by the Kruskal-Wallis test. **Figure 3.25 (A). Figure 3.25 (C), (D) and (E)** show piperacillin-specific IFN- γ secretion from selected clones.

- **IL-5, IL-13, perforin, granzyme-B and Fas ligand**

The clones showed a marked increase in the secretion of IL-5 (17.8 ± 20.2 SFU), IL-13 (53.5 ± 64 SFU), perforin (69.4 ± 80.2 SFU), granzyme-B (71.4 ± 49.1 SFU) and Fas ligand (119.3 ± 136.6 SFU) following treatment with piperacillin (**Figure**

3.26 **A)** When all clones were analyzed together, a statistical significance was observed for the secretion of IL-13, perforin and Fas ligand as analyzed by Mann-



Whitney test. Statistical significance was not achieved with the other cytokines as they were secreted by only a portion of the clones.

3.4.4.1 Measurement of the T cell receptors and chemokine receptors expressed on piperacillin-specific clones

3.4.4.1.1 CD4/CD8 receptors expression

14 piperacillin-specific clones were selected and analyzed. CD4 was expressed on 9 clones (64%) and 5 clones expressed CD8 (36%) (**A**).

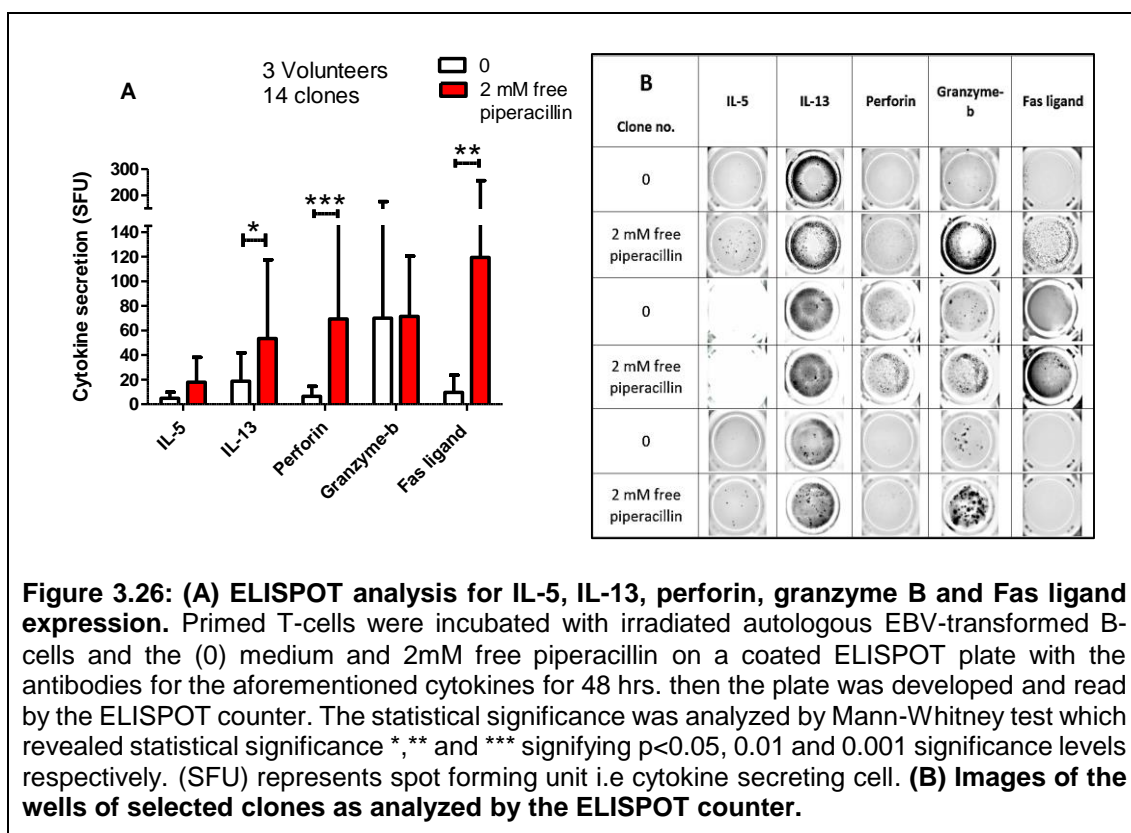
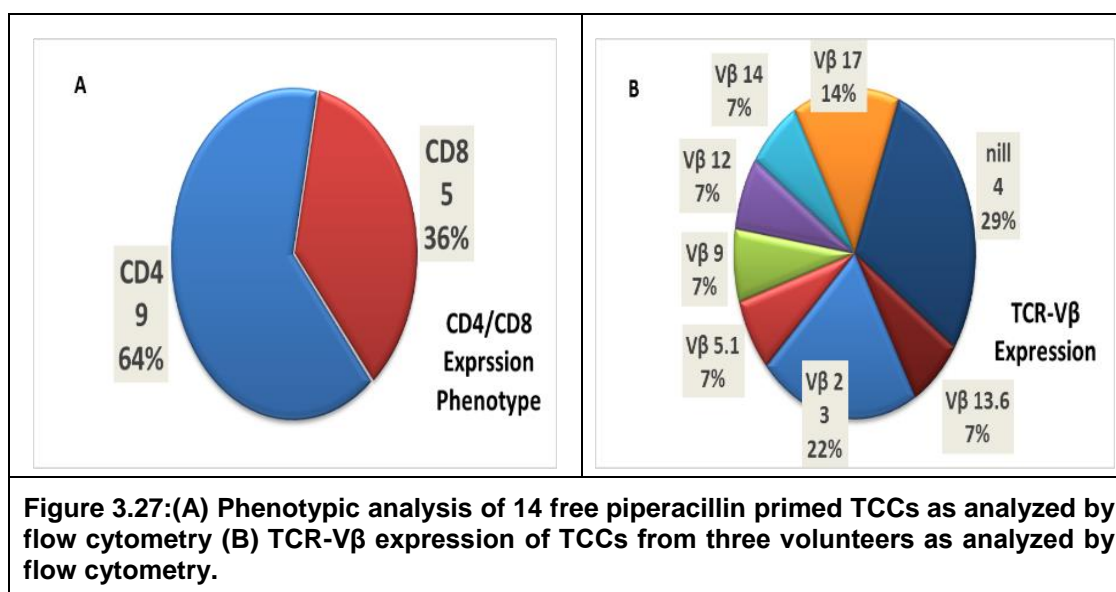


Figure 3.26: (A) ELISPOT analysis for IL-5, IL-13, perforin, granzyme B and Fas ligand expression. Primed T-cells were incubated with irradiated autologous EBV-transformed B-cells and the (0) medium and 2mM free piperacillin on a coated ELISPOT plate with the antibodies for the aforementioned cytokines for 48 hrs. then the plate was developed and read by the ELISPOT counter. The statistical significance was analyzed by Mann-Whitney test which revealed statistical significance *, ** and *** signifying $p < 0.05$, 0.01 and 0.001 significance levels respectively. (SFU) represents spot forming unit i.e cytokine secreting cell. **(B) Images of the wells of selected clones as analyzed by the ELISPOT counter.**

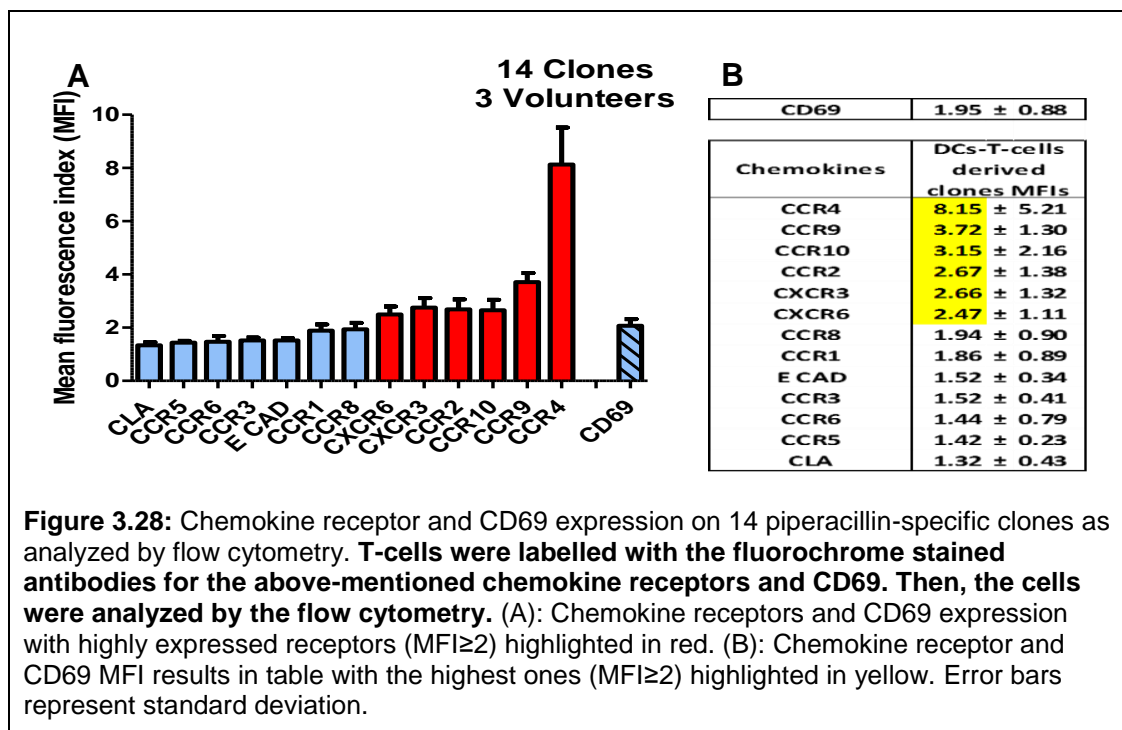
3.4.4.1.2 TCR-V β expression

14 selected clones were labelled and analyzed for TCR-V β expression. The clones showed a diverse pattern of TCR-V β expression (**B**).



3.4.4.1.3 Chemokine receptors and CD69 analysis

14 selected clones were analyzed for chemokine receptor and CD69 expression by flow cytometry. There was a high expression ≥ 2 mean fluorescence index (MFI) for the following chemokines receptors (**Figure 3.28**): CCR4 (8.15 ± 5.21 MFI), CXCR3 (2.66 ± 1.32 MFI), CCR2 (2.67 ± 1.38 MFI), CCR9 (3.72 ± 1.3 MFI), CCR10 (3.15 ± 2.16 MFI), CXCR6 (2.47 ± 1.11 MFI) CD69 the expression was (1.95 ± 0.88).



3.5 Discussion

Idiosyncratic drug reactions are a major concern for the health authorities. Furthermore, treatment of patients with reactions represents a significant financial burden. Regarding β -lactam-induced idiosyncratic drug reactions and particularly piperacillin, T-cells are known to be the main effector cells. Previous research on piperacillin has shown that piperacillin-specific T-cells could be isolated from blood of piperacillin hypersensitive patients (Whitaker et al., 2011).

3.5.1 Activation of T-cells isolated from blood of piperacillin hypersensitive patients with CF

This chapter initially focused on the isolation and characterization of piperacillin-specific TCCs from blood of hypersensitive patients. Six patients in total were analyzed. They varied in terms of the number of piperacillin-specific TCCs generated. For example, almost 200 clones generated from one patient were activated with piperacillin, whereas with others the number of drug-specific clones was 10-fold lower. Finally, we failed to generate clones from one patient classified clinically as piperacillin hypersensitive. This might be due the low number of clones that were expanded from the initial seeding plates (i.e. only 59 clones were tested for reactivity). Thus, we can assume that the yield of clones from each patient PBMCs is patient specific and may be related to the actual number of piperacillin-specific cells in blood of each patient which could be variable between different patients depending on many **drug related** or **patient related** factors (discussed in detail in thesis introduction).

Drug related factors: include dose, frequency and duration of treatment.

Higher dose or more frequent or longer duration would increase the chance of developing hypersensitivity reaction and consequently the number of piperacillin specific T-cells in blood or skin (Thong and Vervloet, 2014).

Patient related: include age: the frequency of hypersensitivity increases with increased age due to cumulative exposure over many years.

Sex: drug hypersensitivity reactions are more common in females.

Concurrent medical illness: asthma or presence of other infections like EBV or HIV (Sullivan and Shear, 2001) increases the chance of hypersensitivity development.

Presence of multiple drug allergy syndrome: increases the chance of hypersensitivity development (Thong and Vervloet, 2014).

HLA association: For some drugs there is an association between the drug hypersensitivity reactions and the HLA phenotype of the patient e.g. Abacavir reactions association with HLA-B*5701 (odds ratio 960) (Mallal et al., 2008), carbamazepine association with HLA-B*1502 in Han Chinese (odds ratio 2504) (Chung et al., 2004). The high frequency of hypersensitivity means it is unlikely that susceptibility is linked to a specific HLA allele. In fact, unpublished data from our laboratory screening HLA alleles expressed on 250 patients with CF revealed no obvious HLA expressed by the hypersensitive patient cohort. It is still possible that piperacillin-derived antigens are preferentially presented to T-cells on a range of HLA alleles; however, these would be difficult to identify in genetic studies.

Pharmacokinetic and pharmacodynamic state of the patient: Although each patient in this study received the same drug treatment regimen, it is likely that they are exposed to different concentrations of free drug and bound protein adduct. This is due to the inter-individual variation in the pharmacokinetic and pharmacodynamic state that would result in diverse plethora of reactions to the same drug.

As stated in most published papers investigating T-cell responses to drugs, it was decided to choose ≥ 2 stimulation index (SI) as a positive response to piperacillin (Pichler, 2014). The isolation of drug-specific clones from 5/6 patients is very close

to previous research that detected piperacillin specific lymphocyte proliferative responses in about 75 % of patients (Whitaker et al., 2011).

The results shown in (**Figure 3.3**) show the optimal dose for activating T-cells with free piperacillin was 2mM. Clinically, the normal peak serum concentration for piperacillin is 389-484 µg/mL with a 4 g I.V. dose of piperacillin (ARUP, 2016). Thus, the dose that we give in vitro is 2-2.6 times above the clinical dose. It is common in in vitro cultures to use higher doses than found in vivo due to the different environment drug exposure and antigen presentation in vitro: culture media constituents and the absence of concomitant infections which make cells in vitro less sensitive to drugs (Huntjens et al., 2006; Sande and Zak, 1999).

SMX-NO, an oxidative metabolite of SMX was used as a structurally irrelevant antigen to test the specificity of the piperacillin-specific clones. SMX causes a high incidence of cutaneous hypersensitivity reactions and SMX-NO-responsive T-cells have been identified in all hypersensitive patients that have been studied to date (Elsheikh et al., 2010; Elsheikh et al., 2011). The piperacillin-responsive clones were not activated to proliferate in the presence of titrated concentrations of SMX-NO.

Flow cytometry data has shown that the majority of clones were CD4+ (n=79, 92%) while a few displayed either a CD8+ (n=4, 5%) or CD4+CD8+ (n=2, 3%) phenotype. The piperacillin-specific clones also secreted high amounts of IFN-γ and Th2 cytokines in response to serially diluted doses of free piperacillin. The mixed Th1 and Th2 cytokine profile suggested that molecules released from these clones will support the recruitment and activation of other immune cells that participate in the hypersensitivity reaction. To investigate whether the piperacillin-specific T-cells might cause tissue injury directly, the secretion of cytolytic molecules was also measured using ELISPOT. The activated clones were found to secrete granzyme-B and perforin, which are able to induce apoptosis in target cells. High expression of perforin and granzyme-B has been reported previously with T-cells from patients with drug-induced skin reactions (Yawalkar et al., 2001)

especially, non-immediate allergic reactions to drugs (Torres et al., 2009). The presence of cytolytic CD4+ T cells suggests a need to revise the classical Th1/Th2 T-cells model to cover wider and more conventional roles played by the T-cells. This would pave the way for a better understanding of the T-cell immune response in drug hypersensitivity reactions.

For CD4+ TCCs, the TCRs interact with piperacillin presented on MHC class II molecules whereas for CD8+ TCCs the TCRs interact with piperacillin presented on MHC class I molecules. This was demonstrated previously using blocking antibodies for the different MHC molecules (El-Ghaiesh et al., 2012). Double positive T-cells are present normally in human blood and are known to respond to MHC I or II restricted antigens which culminate the Th1/Tc profile (Nascimbeni et al., 2004). It is unknown if there is a role for cross presentation in their activation. Extensive research on delayed hypersensitivity reactions to drugs has shown a compartmental distribution of T-cell subtypes. CD4 cells are predominantly in the dermis and blood while the CD8 cells predominantly are in the inflamed epidermis (Friedmann et al., 1994). This suggests that CD8+ T-cells might be the major mediators of certain drug hypersensitivity reactions. However, several studies have shown an increased number of CD4+ T-cells in blood and skin of hypersensitive patients especially in cases of maculopapular exanthema (MPE), which is the predominant clinical presentation in piperacillin hypersensitivity. Thus, it is possible that CD4+ T-cells are the mediators of milder forms of cutaneous hypersensitivity, while CD8+ T-cells are involved in bullous exanthema like SJS and TEN (Hari et al., 2001).

The cells that we used to generate the free piperacillin-responsive clones are PBMCs (i.e. blood derived). Thus, by linking these studies with the T-cells isolated from skin (an on-going project in the CDSS), it will be possible to obtain a detailed assessment of the drug-specific skin-resident and migrating T-cells that are generated in hypersensitive patients.

Apart from the cytolytic actions of T-cells (i.e., secretion of perforin, granzyme-B and Fas ligand), T-cells play a pivotal role in mediating the hypersensitivity reactions by secreting cytokines that act as chemo-attractants and inducers for other inflammatory cells and keratinocytes. Moreover, T-cells express chemokines receptors which determine the tissue they will migrate towards. First of all, IFN- γ exerts a series of actions including activation of macrophages with the resultant eczema (type IVa response according to the revised Gell and Coombs classification) (Torres et al., 2009). IFN- γ secretion with TNF α and T-bet expression (Th1 cytokines) has been shown to be associated with more severe reactions (as in SJS/TEN) (Cornejo-Garcia et al., 2007). Th2 cytokines i.e.IL-5, IL-13, play an important role in triggering B-cells to produce antibodies especially IgE and IgG. Moreover, Th2 cytokines have a stimulatory effect on mast cells and eosinophils (Torres et al., 2009). Th2 cytokines have previously been shown to participate in MPE, DRESS and bullous exanthema and airway hyper-responsiveness in the form of asthma or rhinitis (Torres et al., 2009;Pichler, 2007). Collectively, the immunological mechanisms and the clinical features implicated above are classified under type IVb drug hypersensitivity according to the revised Gell and Coombs classification (Pichler, 2007).

Flow cytometry revealed that TCR-V β expression in the piperacillin-specific clones was diverse. No dominant expression by one TCR-V β was observed. Several studies have previously explored the involvement of specific TCR-V β s in drug hypersensitivity reactions. Notably, carbamazepine induced SJS/TEN has been found to be associated with TCR-V β 11 in 84% of hypersensitive patients but none of the tolerant volunteers. T-cells expressing TCR-V β -11 could be detected in the blister fluid of the SJS/TEN and the blood of carbamazepine hypersensitive patients. Moreover, utilising anti V β -11 prevented the activation of T-cells (Ko et al., 2011). On the other hand, abacavir hypersensitivity shows a diverse pattern of TCR-V β expression (Illing et al., 2012).

The piperacillin reactive clones expressed high levels of the following chemokines receptors: CCR4, CCR9, CXCR3, CCR10, CCR2 and CCR8. CCR4, CXCR3 and CCR10 are receptors involved in the homing of immune cells towards skin (Restifo et al., 2013). This provides an explanation for why the skin is targeted in piperacillin hypersensitive patients. CXCR3 and CCR2 are expressed by Th1 T-cells while CCR3, CCR4 and CCR8 are expressed mainly by Th2 T-cells (Kim et al., 2001), which aligns with the secretion of Th1 and Th2 cytokines by the clones. CCR9 is a marker on gut-homing T-cells (Agace, 2008). It has a role in the development of small and large intestinal inflammation. Moreover, CCR9 antagonists have been found effective in ulcerative colitis treatment (Bekker et al., 2015). Intestinal involvement in case of piperacillin hypersensitivity is relatively common (0.9%) which manifests in the form of diarrhoea, nausea, vomiting and abdominal pain (Rxlist.com, 2015). However, its mechanism involves disruption of the intestinal flora (Barbut and Meynard, 2002). Thus, the role of CCR9 in piperacillin hypersensitivity is not clear. It is possible that the receptor expression might be involved in the migration of T-cells to organs other than the skin, but this topic requires further investigation. Another important role for CCR9 is that it promotes the migration of T-cells to the liver and it has been shown to be highly expressed in cases of primary sclerosing cholangitis (Eksteen et al., 2004). Liver involvement in piperacillin hypersensitivity has been rarely reported.

CD69 is a commonly used marker to measure the activation status of T-cells. This marker has been shown to increase robustly in response to TCR triggering (Porebski et al., 2011). Expression of CD69 by CD4⁺ memory T-cells is critical for their migration and persistence in bone marrow environment (Schoenberger, 2012). Bone marrow is an important secondary lymphoid organ that acts as reservoir for the memory T-cells (CD4⁺ and CD8⁺) in addition to other types of cells including naïve T-cells, DCs and B-cells which interact together to mount long-term immune responses (Di Rosa and Pabst, 2005).

3.5.2 Priming of piperacillin-specific T-cell responses from healthy drug-naïve volunteers

Methods have recently been developed to study the drug-specific priming of naïve T-cells from healthy volunteers. The assay relies on the initial co-culture of highly purified naïve T-cells with autologous dendritic cells and drug for around 8 days. The primed T-cells are then restimulated with a second batch of dendritic cells and drug and antigen-specific responses can be assessed through measurement of proliferation and/or cytokine release (Faulkner et al., 2012; Richter et al., 2013).

Importantly, this assay (DC-T-cell priming) was established with the drug metabolite antigen SMX-NO and the priming of naïve T-cells to β -lactam antibiotics has not been investigated. T-cells primed to free piperacillin showed a dose-dependent proliferative response following restimulation. Moreover, the primed cells do not react with SMX-NO denoting that these cells are sensitive and specific to free piperacillin. The concentration of piperacillin required to activate newly primed T-cells and T-cells from hypersensitive patients was the same.

For ELISPOT assays, IFN- γ cytokine was chosen since it reflects the Th1 T-cell response while the IL-5 and IL-13 reflects the Th-2 T-cell response. Free piperacillin primed T-cells were found to secrete IFN- γ when they were activated with piperacillin, but not SMX-NO. Once successful priming had been established, the secretion of other cytokines was assessed. IL-5 and IL-13 secretion by the primed T-cells to free piperacillin was low. However, the detection of Th1 and Th2 cytokine from piperacillin-specific T-cells from healthy volunteers coincides with that of the clones generated from the hypersensitive patients.

In general, the phenotypic profile of pre-priming and post-priming T-cells shows mild predominance of CD4⁺ over CD8⁺ T-cells. However, the percentages of both the CD4⁺ and the CD8⁺ T-cells decreased post-priming due to the marked increase in double positive (CD4⁺CD8⁺) and double negative (CD4⁻CD8⁻) T-cells post-priming. The rise in the percentage of double positive and double negative

CD4⁺ CD8⁺ may be regarded as a consequence of the priming process that leads to disruption of the T-cell receptors leading to their hyper or hypo expression. The double positive CD4⁺CD8⁺ T-cells constitute 1-3% of the T-cells in the peripheral blood of healthy individuals (Blue et al., 1985). The priming technique entails interaction with the antigen presenting cells (DCs) which exert a stimulatory effect on the T-cells. Such an interaction has been found to be responsible for:

1. The expression of CD4 receptors on CD8 T-cells (Yang et al., 1998).

Interestingly, the presence of CD4⁺ T-cells by itself is an important triggering factor promoting the expression of CD4⁺ on CD8⁺T-cells. It has been suggested that these cells are a distinct population of cells and not an artefact from the classical CD4⁺ cells (Sullivan et al., 2001).

2. The expression of CD8⁺ receptors on CD4⁺ T-cells.

Antigenic stimulation plays an important role on the development of these cells as shown by the sharp rise of CD4⁺CD8⁺ after bacterial or viral infection (Hernández et al., 1998). This CD8 receptor is distinct from the classical CD8 receptor in that the CD8 receptor on the double positive cell is composed of CD8 $\alpha\alpha$ chains (Moebius et al., 1991) while that of the classical CD8 receptor is composed of CD $\alpha\beta$ chains (Norment and Littman, 1988). They do not express CD1 receptor so that they are not immature cells that have escaped the thymus (Zuckermann, 1999). CD4⁺CD8⁺ T-cells can be cloned from peripheral blood CD4⁺ cells and they secrete IL-2 and IFN- γ and provide help for B-cell differentiation (Patel et al., 1989). Moreover, they express $\alpha\beta$ TCR (Moebius et al., 1991). They also express high levels of CD29 which a marker for memory T-cells (Horgan et al., 1992). These cells have been found to increase in number with increasing age signifying the role of longer antigenic stimulation for their development (Zuckermann, 1999).

Theoretically, the CD4 and CD8 co-expression can enhance the reactivity of the T-cell by increasing the avidity of the TCR at the target. This would promote the activation of memory CD4/CD8 double positive cells either by low levels of the antigen or by the presence of antigen presenting cell with MHC class II at the surface at an insufficient level to activate a memory T-cell. The latter situation is likely to happen outside the secondary lymphoid tissues (Zuckermann, 1999).

Double negative T-cells are assumed to originate from thymus and then escape the negative selection and migrate to the periphery which is followed by expansion after antigenic stimulation (Priatel et al., 2001). They constitute 1-5% of T-cells in the peripheral blood and the lymphoid organs in humans (Juvet and Zhang, 2012). A plethora of studies have highlighted the role of double negative T-cells in the development autoimmune diseases e.g. myasthenia gravis, systemic lupus erythematosus (Reinhardt and Melms, 2000). However, to date there is no specific marker to identify this subset of cells and further work is needed to explore their ontogenesis and profile (Juvet and Zhang, 2012). Another possible explanation for the increase in the double negative T-cells is the activation of the $\gamma\delta$ T-cells (which lack both CD4 and CD8 expression). These cells are normally present in the blood of healthy individuals in a low proportion 1-10% (Pinheiro et al., 2012).

The marked increase of double positive and double negative cells represents an interesting finding that requires further work to investigate its precise nature and the role of these T-cells in disease pathogenesis.

The possible number of TCR-V β combinations in human is 2×10^7 (Laydon et al., 2015). For the sake of simplicity the these TCR-V β s were classified by merging them into 24 families based upon sequence similarity (Wei et al., 1994). This would allow us to detect 70-75% of the total TCR-V β repertoire (van den Beemd et al., 2000). To date, there are two methods for studying and analysing the TCR-V β : (1) flow cytometry and (2) PCR techniques. In comparison to PCR, the flow cytometry is much quicker and cheaper with the privilege of allowing the

researcher to analyse the V β repertoire within the T-cell subsets in a highly precise manner. Moreover, it can be utilized as a screening tool for detecting large T-cell expansion with a single TCR-V β . Flow cytometry permits the selection of different T-cells subsets by combining TCR-V β mAbs with other markers (van den Beemd et al., 2000).

Based on this discussion, flow cytometry was chosen to study the TCR-V β expression on piperacillin-primed T-cells. There was a high variation (skewing) in the TCR-V β expression from prepriming to post priming with some TCR-V β s increasing and others decreasing. However, when data from the individual donors were compared, variation of the mean change ratio (post priming/prepriming) for some TCR-V β was observed. The following TCR-V β showed a marked increase: V β -9, V β -13.2, V β -18 and V β -4. By comparing this result with the levels of TCR-V β in healthy controls from another study (van den Beemd et al., 2000), we can notice that there is a marked increase from the average TCR-V β of healthy naïve volunteers as shown in **Table 3.11**:

Table 3.11: Comparison of selected high TCR-Vβ post priming (in out study) with their expression in healthy volunteers as shown in (van den Beemd et al., 2000)					
	Postpriming		Healthy controls		
	Mean	Standard deviation	Mean	Standard deviation	change percentage
Vb 9	3.1	1.7	2	3.5	155.33%
Vb 18	1.9	0.4	2	3.5	94.33%
Vb 13.2	10.4	1.6	4	7	260.58%
Vb 4	2.9	1.0	1.5	2	191.11%

This profile shows some similarity to that obtained with piperacillin specific clones from hypersensitive patients in that there is expression of V β -9 V β -13.2. It is also important to emphasize that the piperacillin hypersensitive patients suffered from CF. In contrast, the T-cell priming experiments were conducted on healthy volunteers. If the priming experiments were performed on patients with CF, a more

similar profile of V β receptors might have been detected on the drug-specific clones.

The profile of chemokine receptors expressed following priming was similar to the chemokine receptors expressed by piperacillin-specific clones isolated from hypersensitive patients. The patient clones expressed CCR4, CCR9, CXCR3, CCR10, CCR2 and CCR8. In general, the phenotypic profile of the free piperacillin-primed T-cells (in terms of proliferation, cytokines, chemokines receptors, and flow cytometric data) show great deal of similarity to that of the patients-derived clones. This would imply that DC-T-cell priming to free piperacillin could be implemented as a simulation to in vivo priming that occurs in the hypersensitive patients.

3.5.3 Piperacillin-specific T-cell clones generated from PBMCs of healthy naive volunteers

The average percentage of reactive clones that were generated from the naive volunteers after priming was 4.9% (n=40 out of 812 clones). Compared to that of the clones from hypersensitive patients which is 21.9% (n=360 out of 1642 clones) it is clear that it is easier to isolate clones from hypersensitive patients. Thirty piperacillin-reactive clones were selected for phenotyping and functional analysis. Similar to clones from hypersensitive patients, clones generated from volunteers following priming, proliferated in a dose-dependent manner with a maximal response observed at 2mM free piperacillin. The proliferative response of the piperacillin-specific clones was significantly weaker than that of clones from hypersensitive patients. Thus, hypersensitive-patients T-cells seem to have a higher affinity to the specific drug antigen. This may be due to repeated drug exposure for long durations in vivo which would mediate affinity maturation for the specific antigen (Rees et al., 1999). Due to short incubation time with piperacillin in DC-T-cell priming, we do not expect a strong affinity maturation to occur. Practically, we can perceive this in the lower SIs of clones from naïve volunteers as compared with those of hypersensitive patients.

Clones were not activated with the irrelevant antigen SMX-NO. Fourteen clones secreted IFN- γ following piperacillin treatment. A number of these clones secreted Th2 cytokines alongside cytolytic molecules. These cytokine data are similar to that of the clones generated from the piperacillin hypersensitive patients and also similar to the cytokines results of the DC-primed T-cells in that it shows a mixed picture of Th1 and Th2 cytokines. Higher numbers of CD4+ clones were isolated from hypersensitive patients compared with the DC-T-cell priming. All of the clones expressed a CD45RO+ve memory phenotype. Fourteen clones analyzed for their TCR-V β expression using the flow cytometry showed that TCR-V β expression profile was diverse, similar to that expressed by the patients clones.

In conclusion, the data described in this chapter describe the isolation and characterization of piperacillin-specific TCCs from the blood of hypersensitive patients. Clones expressed a mixed Th1 and Th2 phenotype and secreted cytolytic molecules that might cause tissue injury in patients. Furthermore, it was possible to prime naïve T-cells to piperacillin using a recently established DCs-T-cell co-culture system. Cloning revealed that the functionality of the drug-primed T-cells was similar to the T-cells isolated from hypersensitive patients.

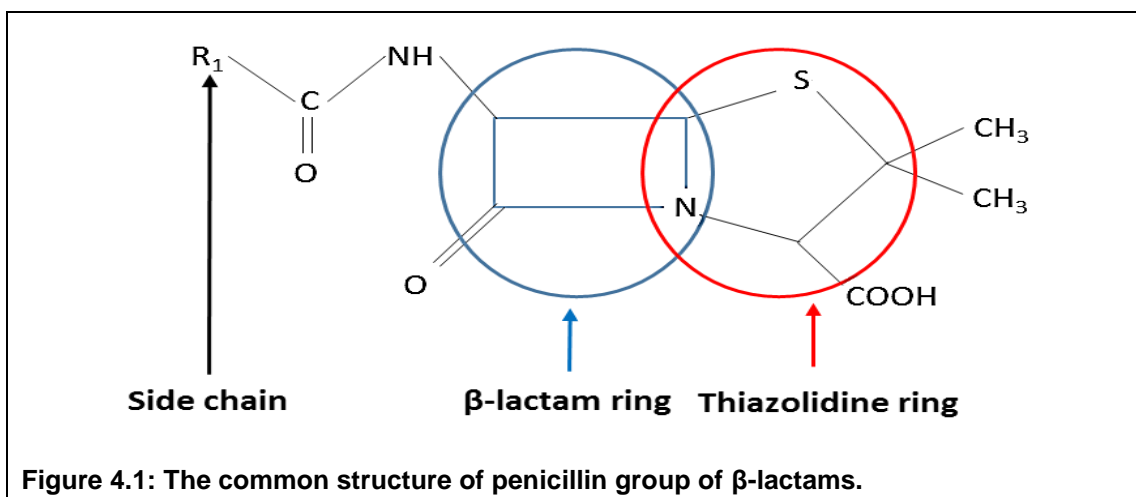
Chapter Four

Conjugated Piperacillin

4 Characterization of the T-cell response to piperacillin HSA adducts

4.1 Introduction

β -lactam antibiotics have a common structure that is composed of β -lactam core ring which mediates the inhibitory effect on the bacterial cell wall and side chains. In addition to these, there is a five membered thiazolidine ring in the penicillin group of antibiotics (**Figure 4.1**). β -lactams form adducts with protein through an irreversible covalent bond and as such represent an ideal drug class to investigate the relationship between hapten protein binding and induction of an immune response.



For adduct formation, the β -lactam ring is targeted by reactive amino acid residues in protein. The initial nucleophilic attack leads to opening of the β -lactam ring and binding of the penicilloyl group. Drug-protein adducts also derive from spontaneous conversion the parent drug into penicilloic acid and penicilloate (Levine and Ovary, 1961).

Through evolution of bioanalytical technologies, mainly protein mass spectrometry, it has been possible to probe the nature of the drug protein

interaction in greater detail. β -lactam antibiotics bind to extracellular protein with a degree of selectivity (Ariza et al., 2014; Ariza et al., 2012).

Furthermore, when cellular and serum protein is separated by an intact plasma membrane, β -lactam antibiotics bind to serum proteins, in particular human serum albumin (HSA) (Jenkins et al., 2013; El-Ghaiesh et al., 2012; Whitaker et al., 2011; Meng et al., 2011). Mass spectrometric analysis of relative peak intensity suggests that adduct formation on albumin is time and concentration-dependent and under physiological conditions. Modifications are detected at fewer than 10% of available nucleophilic sites (Whitaker et al., 2011). Lysine residues in HSA are the only amino acid residues modified by β -lactam antibiotics. The selective binding interaction does not only relate to pKa and therefore reactivity of the side-chain amino group as most adducts form at or around Sudlow sites, which are hydrophobic pockets involved in the non-covalent binding of drugs and endogenous molecules (Sudlow et al., 1975). A recent study exploring the ceftriaxone/albumin interaction found that the drug reacts spontaneously via van der Waals forces and hydrogen bonds and alters the proteins secondary structure (Pan et al., 2012). Collectively, these data suggest that non-covalent binding of β -lactam antibiotics with albumin partly determines the site selective covalent binding interaction and adduct formation.

T-cells from patients with hypersensitivity are activated with β -lactam-HSA adducts (Brander et al., 1995). Furthermore, synthetic designer peptides modified with penicillin haptens stimulate T-cell proliferative responses (Padovan et al., 1996) indicating that protein processing likely generates haptenic antigenic determinants. It is well-known that antigen dose plays a crucial role in the severity of the hypersensitive phenotype (Aguilar-Pimentel et al., 2010; Long et al., 2011) and in determining the characteristics of the responding T-cell repertoire (Oling et al., 2010). The use of **liquid chromatography coupled with mass spectrometry (LC-MS)** together with suitable internal standards is now one of the most widely used techniques for quantification purposes. Therefore, this study

aimed to utilize mass spectrometric methods to quantify the level of β -lactam protein binding in patients and to define the association between the adduct exposure and the drug-specific T cell response. The study focuses on piperacillin, an intravenous β -lactam antibiotic often administered to patients with cystic fibrosis for the treatment of recurrent respiratory infections. Previous research has shown the presence of piperacillin hapten-specific CD4⁺ T-cells in approximately 75% of hypersensitive patients (El-Ghaiesh et al., 2012;Whitaker et al., 2011). Furthermore, studies described in chapter 3 characterized the cellular phenotype and functionality of TCCs responsive to free piperacillin. Thus, piperacillin represents the ideal candidate to investigate the quantitative relationship between adduct formation and drug immunogenicity.

HSA was implemented as a model protein to quantify piperacillin binding in patients and to study the level of modification needed for the activation of specific T-cells.

4.2 Aims of the study

Great progress has been made over the last decade in defining the role of drug-protein binding in hypersensitivity reactions. However, our knowledge is still far from complete. There are questions that remain unanswered; some of these are detailed below:

1. How to characterise and compare the levels of piperacillin-HSA adducts formed in vivo and in vitro?
2. Is it possible to generate clones reactive against synthetic piperacillin-HSA adducts from PBMCs of hypersensitive patients and/or healthy volunteers?
3. What is the role of APCs in mediating the T-cell response to piperacillin protein adducts?
4. What are the characteristics of the clones in terms of sensitivity, antigen specificity and cytokine secretion?

5. Does the concentration of piperacillin-HSA adduct required to trigger T-cell activation correlate with the levels of adduct formed in plasma of hypersensitive patients?
6. What is the phenotype of clones?
7. Are clones responsive against free piperacillin activated with synthetic piperacillin-HSA adducts?

In accordance with the aforementioned questions, the aim of this study is detailed below:

- **To explore the role of drug conjugation in the activation of T-cells from hypersensitive patients and compare the level of adduct needed to activate T-cells with that formed in patients. To address this aim, we have**
 1. Characterized piperacillin-albumin binding sites by mass spectrometry.
 2. Generated synthetic piperacillin HSA adducts.
 3. Generated T-cells responsive against piperacillin-HSA adducts and explored the nature of the induced response focussing on mechanisms of T-cell activation, TCR V β usage and cytokine release.

*Note: The term **free piperacillin** mentioned in this chapter refers to soluble piperacillin available in the medium during T-cells assays. During this time (2-3 days) piperacillin-HSA adducts will form and these have been characterized.*

4.4 Results

The modified peptide synthesis, piperacillin-HSA synthesis and mass spectrometric analysis was conducted by Drs Xiaoli Meng and Rozalind Jenkins (MRC Centre of Drug Safety Science, University of Liverpool).

4.4.1 Synthesis of piperacillin-modified albumin peptide

Our previous studies have shown the formation of piperacillin-modified lysine residue (Lys541) at low concentrations of piperacillin. While at higher concentrations, there are up to 13 lysine residues found to be modified by piperacillin. Some of these residues are detected in patients plasma like (Lys190, 195, 432 and 541). Thus, we proceeded in developing a technique to synthesize the peptide (ATK(piperacillin)EQLK) which is an amino acid sequence peptide with the modified piperacillin-lysine (Lys541) of HSA. This synthetic peptide is essential to allow us to generate a standard curve enabling us to quantify piperacillin hapten binding at this site in both, the tolerant CF patients plasma samples and the in vitro culture assays.

To quantify drug hapten albumin binding, methods were developed to synthesize a piperacillin-modified HSA peptide incorporating the amino acid sequence around Lys541 (ATK[Pip]EQLK) as described in **Figure 4.3 B**. The synthesis of peptide was performed manually in solution phase using Fmoc strategy employing orthogonal protection strategy. Piperacillin benzyl ester was selectively conjugated to ϵ -amino group of lysine in mild reaction conditions, and the resulting piperacillin-modified lysine was incorporated with relevant amino acid residues, followed by deprotection by using catalytic hydrogenolysis to generate peptide ATK(Pip)EQLK. LC-MS/MS analysis revealed formation of desired peptide. The presence of a doubly charged ion at m/z 676.8, corresponding to the peptide ATKEQLK with an additional mass of 535 amu, demonstrated that a hydrolyzed piperacillin-modified peptide was formed. The peptide sequence was confirmed by a product ion spectrum that generated partial singly charged y and b series

ions. The modification was defined by b3* (peak at m/z 517) and b5* (peak at m/z 774.4), with a mass addition of 216 amu, providing evidence that piperacillin is attached to K541 as shown in **Figure 4.3 C**. Purification of crude peptide products by HPLC afforded peptide ATK(piperacillin)EQLK in 90% purity.

Patients details

Plasma samples were isolated from blood of ten piperacillin tolerant patients with cystic fibrosis prior to piperacillin exposure and immediately after a standard 14-day treatment course (4.5g qds). Plasma was aliquoted and stored at -80°C immediately after isolation for characterization and quantification of piperacillin hapten HSA adducts.

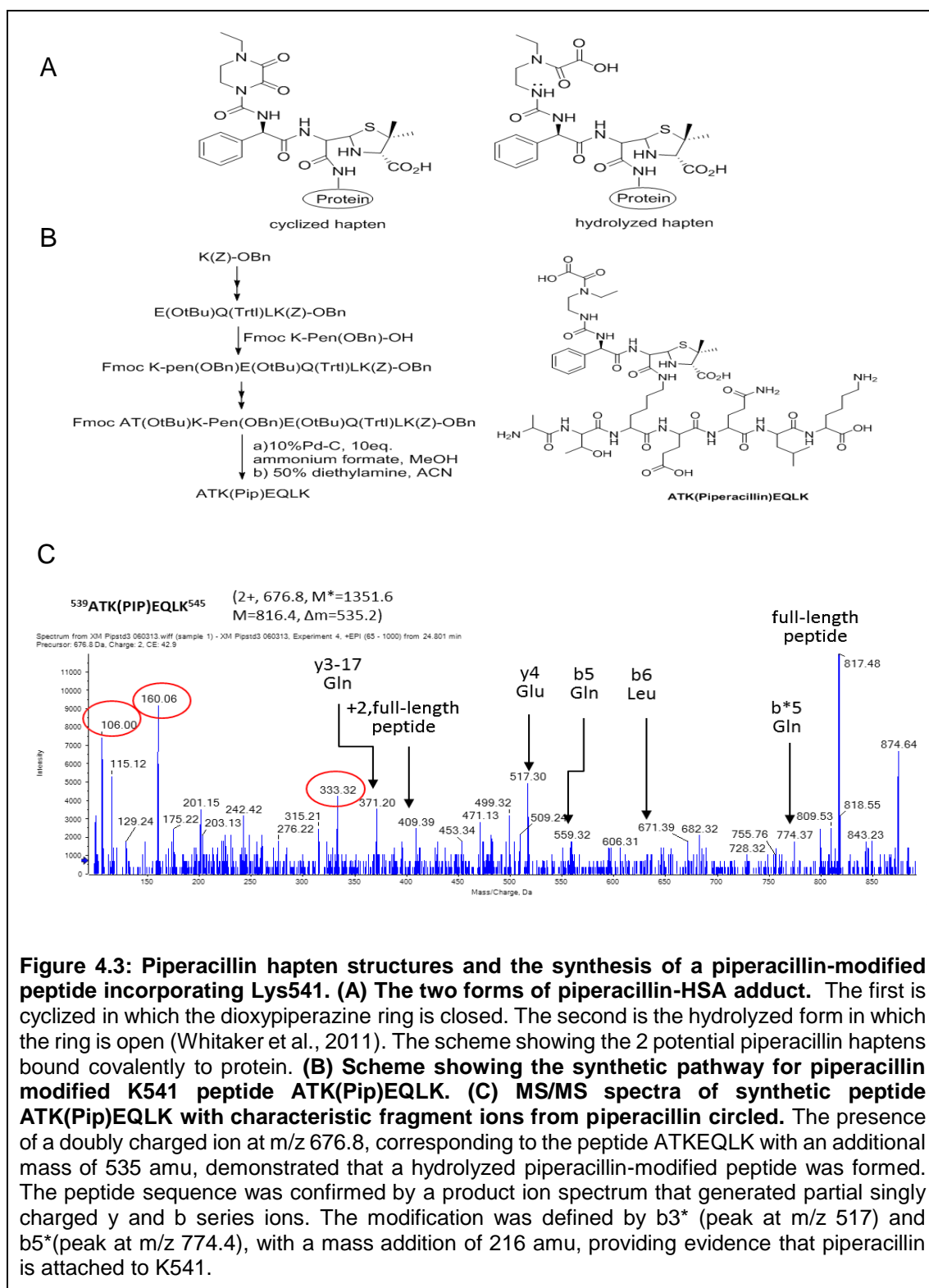
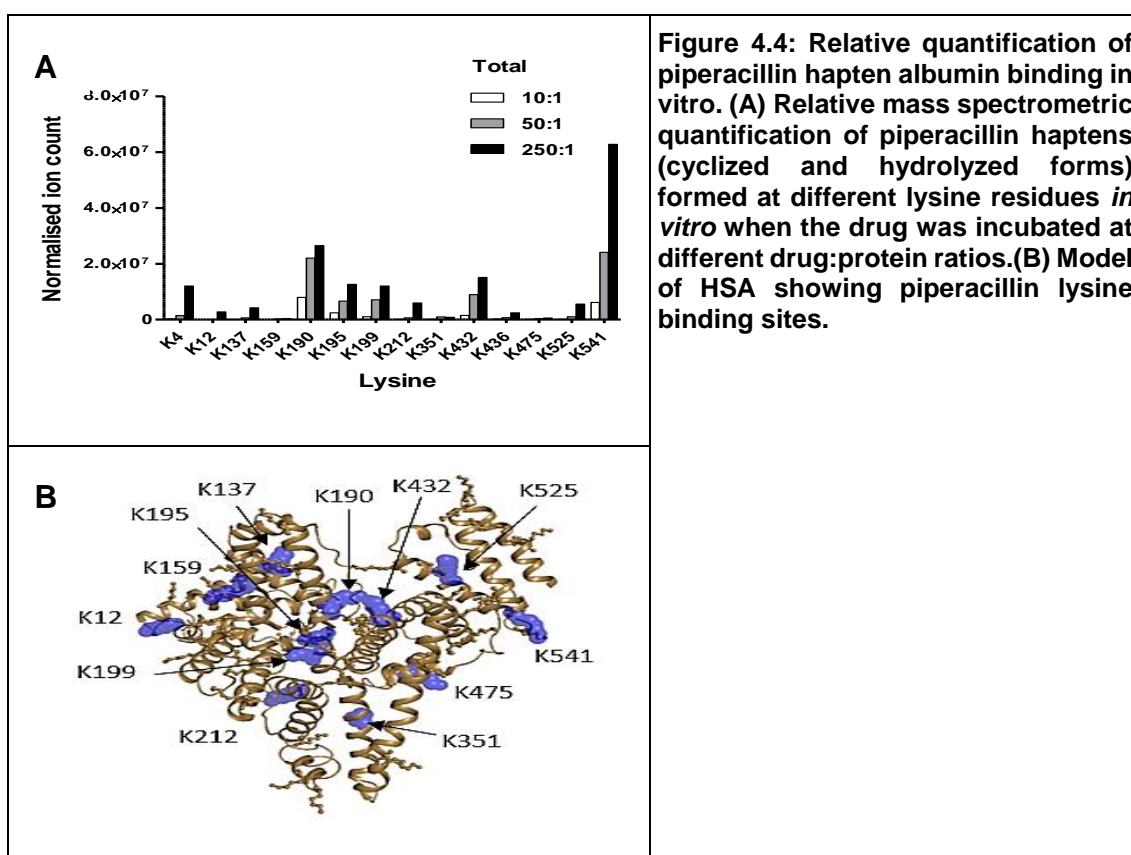


Figure 4.3: Piperacillin hapten structures and the synthesis of a piperacillin-modified peptide incorporating Lys541. (A) The two forms of piperacillin-HSA adduct. The first is cyclized in which the dioxypiperazine ring is closed. The second is the hydrolyzed form in which the ring is open (Whitaker et al., 2011). The scheme showing the 2 potential piperacillin haptens bound covalently to protein. **(B)** Scheme showing the synthetic pathway for piperacillin modified K541 peptide ATK(Pip)EQLK. **(C)** MS/MS spectra of synthetic peptide ATK(Pip)EQLK with characteristic fragment ions from piperacillin circled. The presence of a doubly charged ion at m/z 676.8, corresponding to the peptide ATKEQLK with an additional mass of 535 amu, demonstrated that a hydrolyzed piperacillin-modified peptide was formed. The peptide sequence was confirmed by a product ion spectrum that generated partial singly charged y and b series ions. The modification was defined by b3* (peak at m/z 517) and b5* (peak at m/z 774.4), with a mass addition of 216 amu, providing evidence that piperacillin is attached to K541.

4.4.2 Quantitative analysis of piperacillin-modified albumin in patients

Up to 13 lysine residues were modified with cyclized and hydrolyzed forms of piperacillin hapten at different drug to protein ratios *in vitro* (**Figure 4.4 A**). Using relative quantification based on ionization efficiency, Lys541 was the most frequently modified residue. Lys541 and Lys190 peptides were present at similar level in patients (results not shown). A peptide containing piperacillin-modified Lys541 was therefore used to quantify piperacillin-modified albumin in patients.

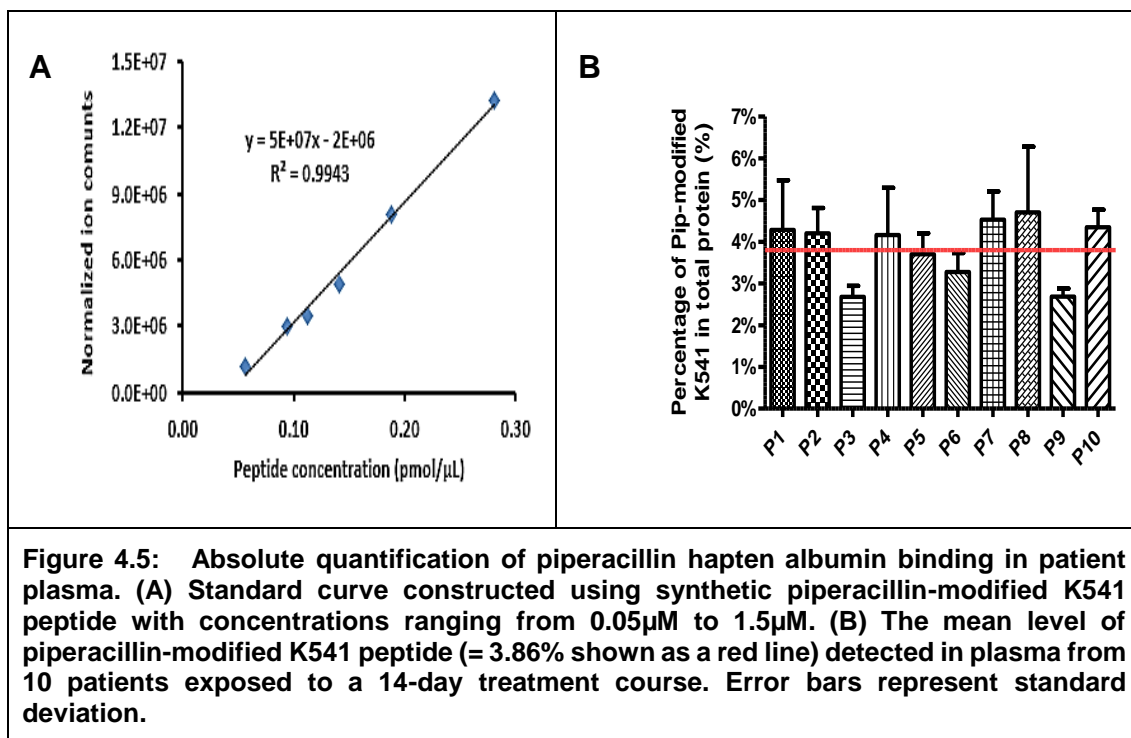
Albumin was isolated from patients plasma samples (n=10) after a 14-day



treatment course by means of affinity column. Following processing the samples were analyzed directly by LC-MS/MS. In contrast to the two hapten structures detected on piperacillin albumin adducts generated *in vitro* (cyclized and

hydrolyzed (**Figure 4.3 A**). The hydrolyzed form was the only hapten detected on albumin Lys residues in patients (results not shown), with tryptic peptides incorporating Lys541 and Lys190 being the dominant sites of modification. A total of four lysine residues, namely Lys190, Lys432, Lys 525, and Lys541, were detected in all patients.

A calibration curve was constructed using the synthetic piperacillin-Lys541 peptide to quantify piperacillin albumin binding in patients (**Figure 4.5 A**); the analyses were conducted on three separate occasions). The level of modification of Lys541 in total HSA ranged from 2.7-4.7% (mean 3.86%), with 6 patients displaying modification levels of 4% or more (**Figure 4.5 B**). Interestingly, the levels of piperacillin-modified Lys541 in some patients were above that formed *in vitro* after a 24 hrs incubation (3.6% for drug-protein molar ratio 10:1 as shown in **Figure 4.6 C**).



4.4.3 Generation and characterization of piperacillin-HSA adducts

Synthetic piperacillin-HSA adducts were characterized by mass spectrometry and western blot. Epitope profiling showed that both cyclized and hydrolyzed hapten were formed; notwithstanding the disparity in the ionisation efficiency of the peptides, piperacillin-modified K541 was the major binding site (**Figure 4.6 A and B**). The levels of hydrolyzed piperacillin-modified K541 elevated with increased concentrations of piperacillin, ranging from 3.6% at the lowest T-cell stimulatory molar ratio 10:1 (discussed later under **heading 4.4.5.2**) to 23.5% at a drug:protein ratio of 250:1 (**Figure 4.6 C**). The concentration-dependent binding of piperacillin was also mirrored using western blotting (**Figure 4.6 D**).

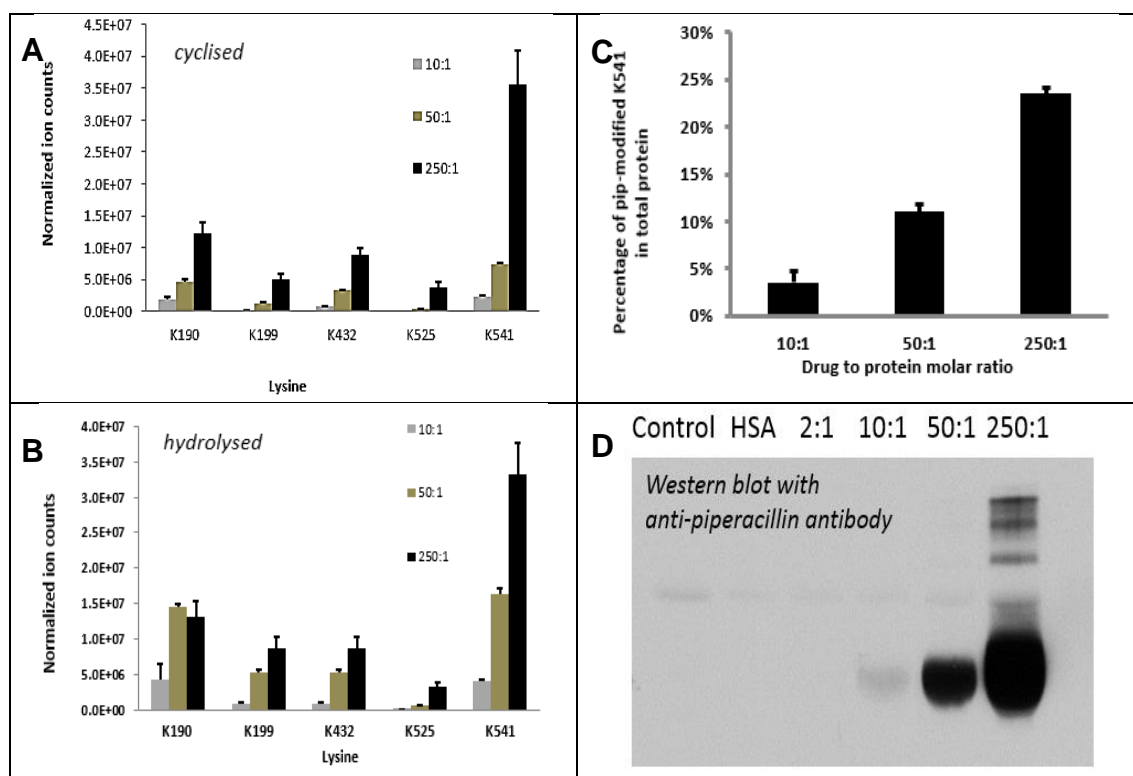


Figure 4.6: Relative quantification of (A) cyclized and (B) hydrolyzed forms of piperacillin hapten generated at different lysine residues on piperacillin albumin adduct (drug:protein ratios of 10:1-250:1). (C) The absolute level of piperacillin-modified K541 peptide detected on piperacillin albumin adduct generated at drug:protein ratios of 10:1-250:1. (D) Western blot analysis of piperacillin albumin adducts. Error bars represent standard deviation.

4.4.4 Characterization of the epitope profile of piperacillin- HSA adducts

After being digested with trypsin and analyzed using mass spectrometry, 13 peptides containing piperacillin were detected out of 59 lysine residues for both forms of piperacillin: the cyclized and the hydrolyzed (**Table 4.1**).

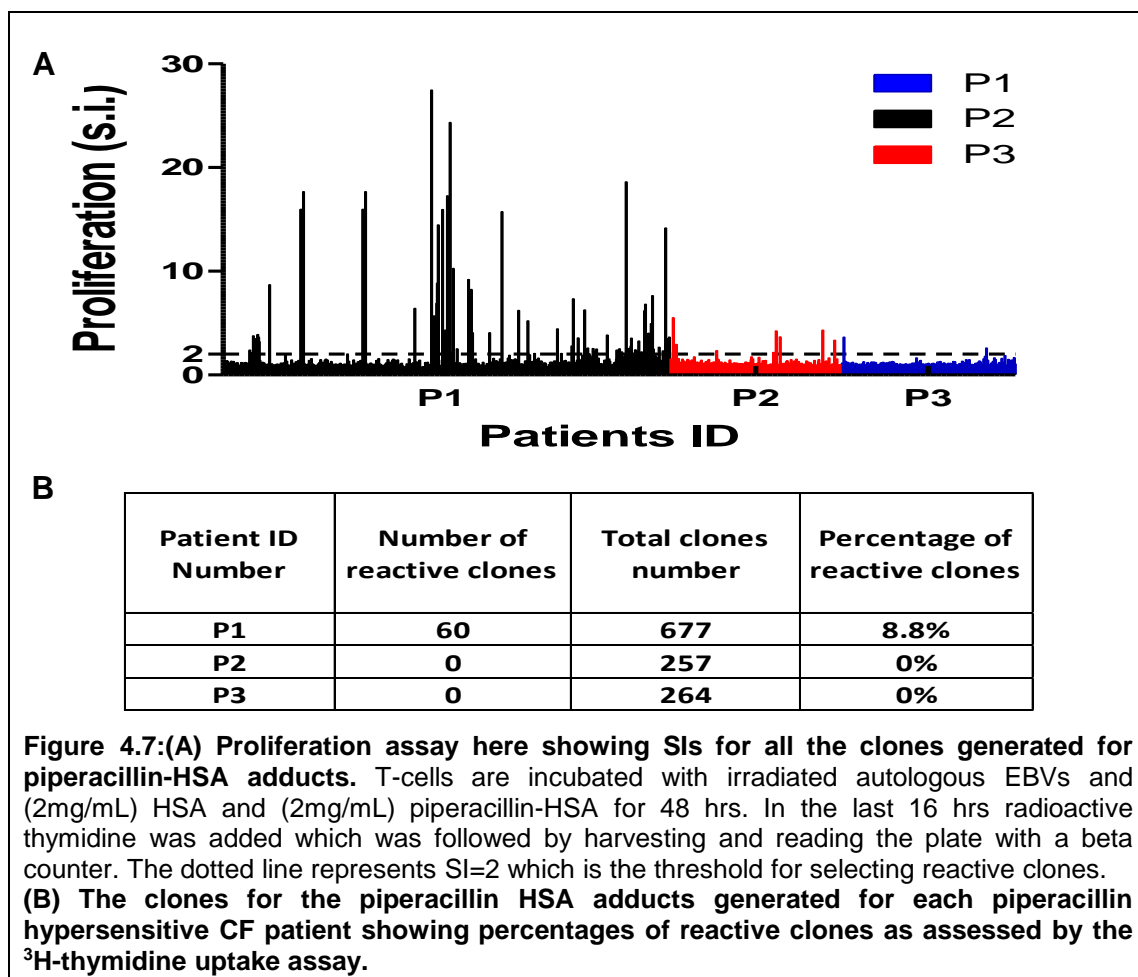
Table 4.1: Lysine residues containing piperacillin after tryptic digestion of piperacillin-HSA and analysis of the resultant peptides using mass spectrometry. *=piperacillin			
Lysine residue	Peptide	Hydrolyzed hapten	Cyclized hapten
4	DAHK*SEVAHR	✓	✓
12	FK*DLGEENFK	✓	✓
137	K*YLYEIAR	✓	✓
162	YK*AAFTECCQAADK	✓	✓
190	LDELRDEGK*ASSAK	✓	✓
195	ASSAK*QR	✓	✓
199	LK*CASLQK	✓	✓
212	AFK*AWAVAR	✓	✓
351	LAK*TYETTLEK	✓	✓
432	NLGK*VGSK	✓	✓
525	K*QTALVELVK	✓	✓
541	ATK*EQLK	✓	✓
545	EQLK*AVMDDFAAFVEK	✓	✓

4.4.5 Characterisation of the response of patient T-lymphocytes to piperacillin adducts

4.4.5.1 Generation of drug-specific T-cell lines and T-cell clones from piperacillin-hypersensitive patients

Three piperacillin hypersensitive patients were chosen (the first three patients illustrated in **Table 3.1**) who have previously been shown to be positive for piperacillin in LTTs (Whitaker et al., 2011) with historical delayed-onset maculopapular exanthema. In every case, the treatment had to be discontinued,

and patients received anti-histamines and/or oral steroids, when clinically indicated. Blood samples were collected after 4 weeks of drug discontinuation and PBMCs were separated. T-cell lines were prepared by incubating the PBMCs in culture with (2mg/mL) of piperacillin-HSA (250:1 molar ratio) for 14 days. Subsequently, clones were generated using the method of serial dilution. Following mitogen-driven expansion, clones were tested using ^3H -thymidine proliferation assay with irradiated EBV-transformed B-cells as antigen presenting cells. 2mg/mL unmodified HSA was used as a comparator against 2mg/mL piperacillin-modified HSA generated with a 250:1 molar ratio. SI ≥ 2 was used as a cut-off point to identify the reactive clones.



60 clones (out of 1198) were found to proliferate actively in the presence of piperacillin HSA conjugate (**Figure 4.7**).

4.4.5.2 Measurement of the reactivity of piperacillin-HSA primed clones to various molar ratios of piperacillin-HSA adduct using ^3H -thymidine uptake proliferation assay

Ten clones were assessed for their reactivity in terms of proliferation to piperacillin HSA adducts (2mg/mL) generated at different molar ratios of drug:protein. Cell culture medium (0) and unmodified HSA (2mg/mL) exposed to the same

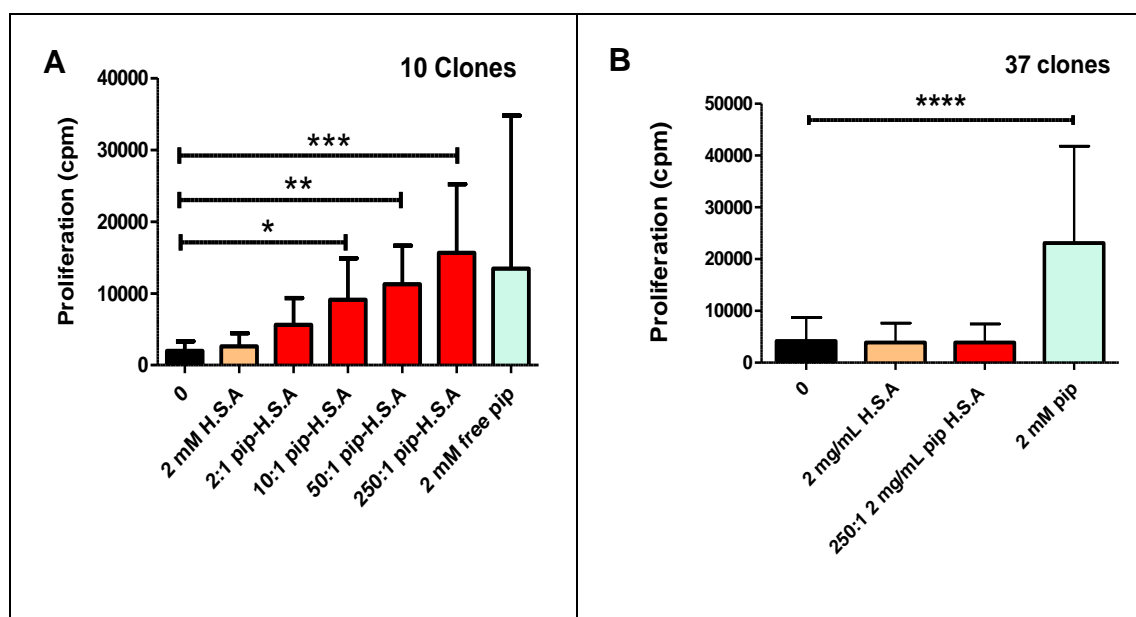
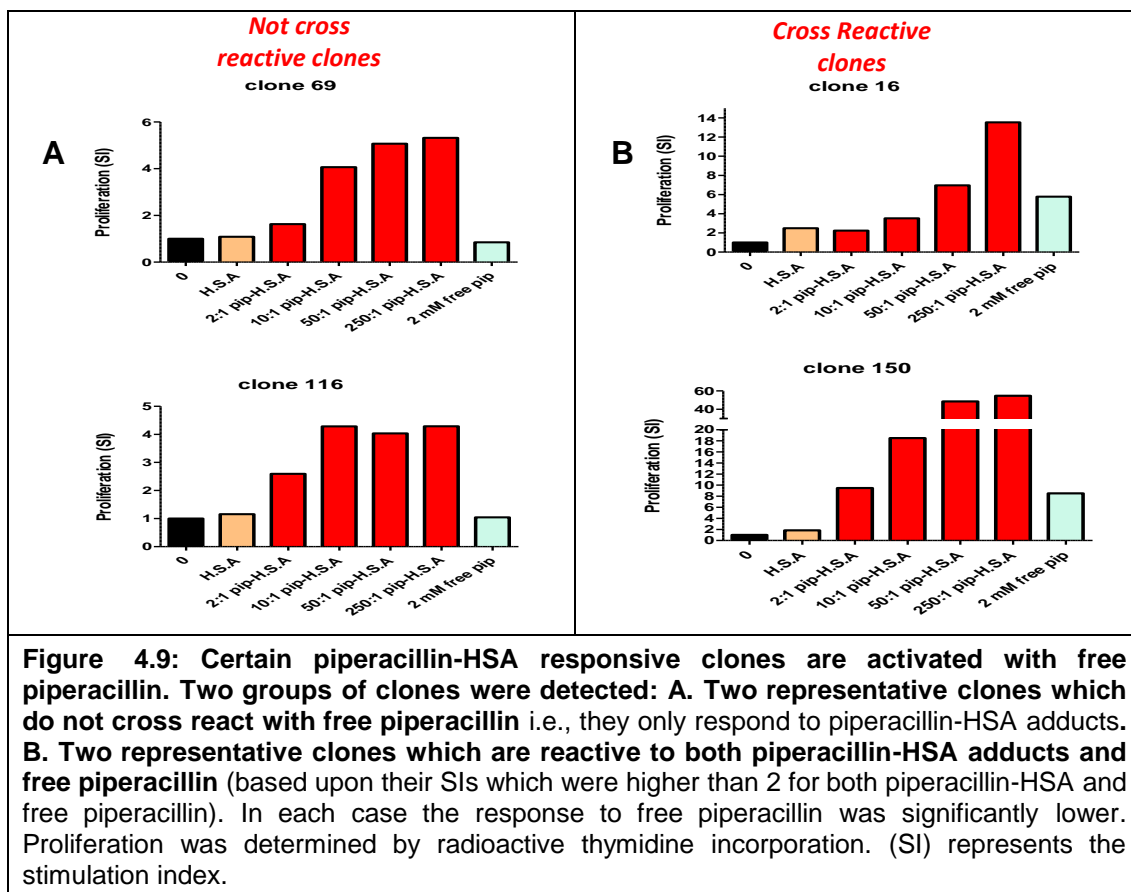


Figure 4.8: ^3H -thymidine proliferation assay for the (A) piperacillin-HSA, (B) free piperacillin primed clones using (2mg/mL) piperacillin-HSA in various molar ratios, (2mg/mL) HSA and (2mM) free piperacillin. T-cells were incubated with irradiated autologous EBVs and (2mg/mL) HSA and (2mg/mL) piperacillin-HSA for 48 hrs. In the last 16 hrs radioactive thymidine was added which was followed by harvesting and reading plate with beta counter. The clones show a dose-dependent response curve. Statistical analysis using Kruskal-Wallis test reveal high statistical significance *, ** and *** signifying $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Error bars represent standard deviation. (cpm) represents the count per minute (i.e. radiation unit).

extraction method as the protein adduct were used as negative controls. Furthermore, clones were cultured with piperacillin at (2 mM) to assess cross reactivity between piperacillin-HSA adducts and the free drug. Clones were all

activated to proliferate with piperacillin-HSA adduct, with the strongest response observed at a 250:1 piperacillin-HSA molar ratio as shown in **Figure 4.8 A**. Statistical significance was observed at a molar ratio of 10:1, 50:1 and 250:1 piperacillin-HSA as analyzed by Kruskal-Wallis test.

Interestingly, some clones displaying reactivity to piperacillin-HSA adducts were activated with free piperacillin, while others were not (**Figure 4.9**). This is despite the fact that piperacillin-HSA adducts are generated spontaneously in the cell culture medium containing free drug.



4.4.5.3 ^3H -thymidine uptake proliferative response of free piperacillin clones to piperacillin-HSA adducts

Thirty-seven clones reactive to free piperacillin, generated from 5 piperacillin hypersensitive CF patients, (see chapter 3) were selected and tested for their cross reactive proliferative response to piperacillin-HSA adducts using ^3H -thymidine uptake proliferation assay.

The results in **Figure 4.8 B** show that these clones have very high reactivity to free piperacillin (2mM) (****: $p < 0.0001$) as analyzed by Kruskal-Wallis test. The clones in general did not show reactivity for piperacillin adducts. Three clones were found to show reactivity for both the free piperacillin and the adducts (**Figure 4.10**).

In total, (50%) of piperacillin-HSA conjugates primed TCCs are cross reactive to free piperacillin while few of the free piperacillin primed TCCs (8.1%) are cross reactive with piperacillin-HSA conjugates as shown in **Table 4.2**.

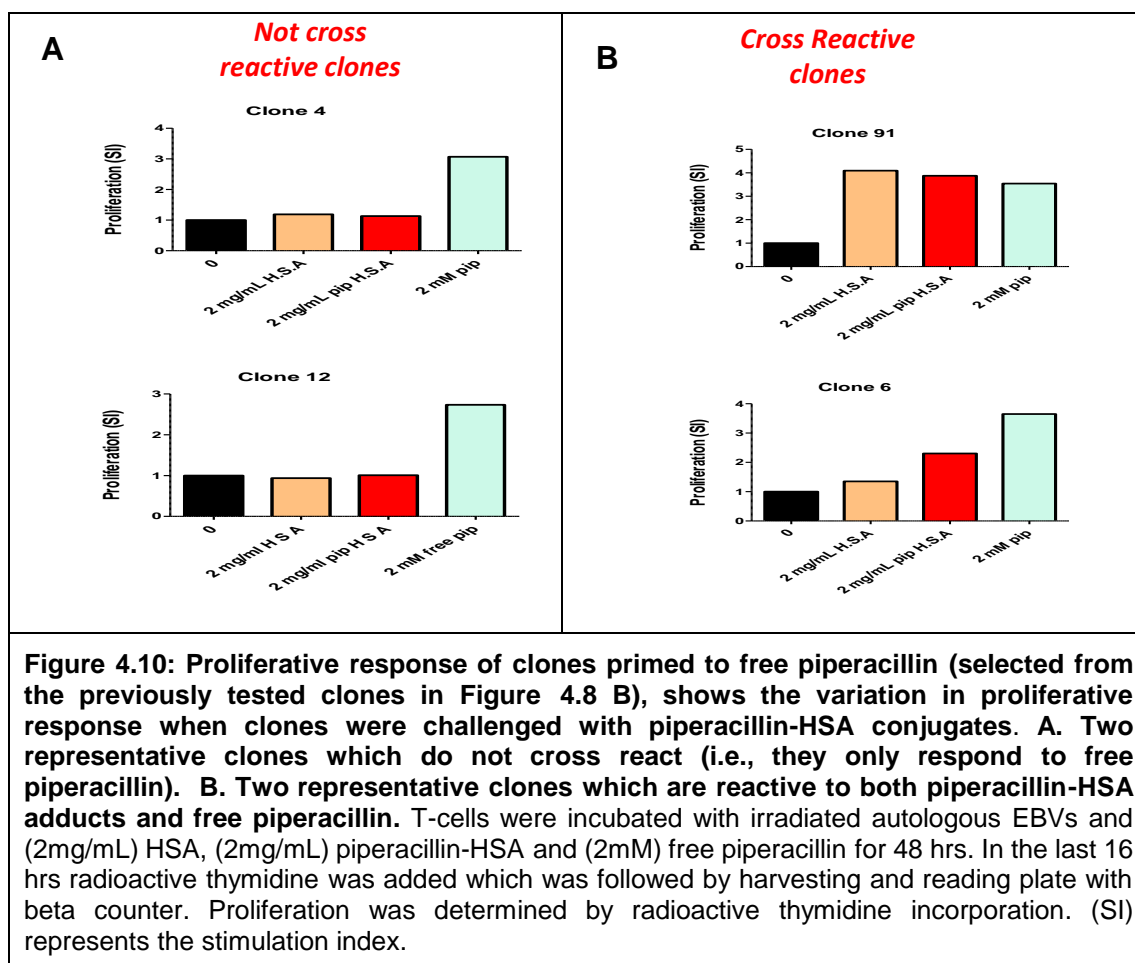


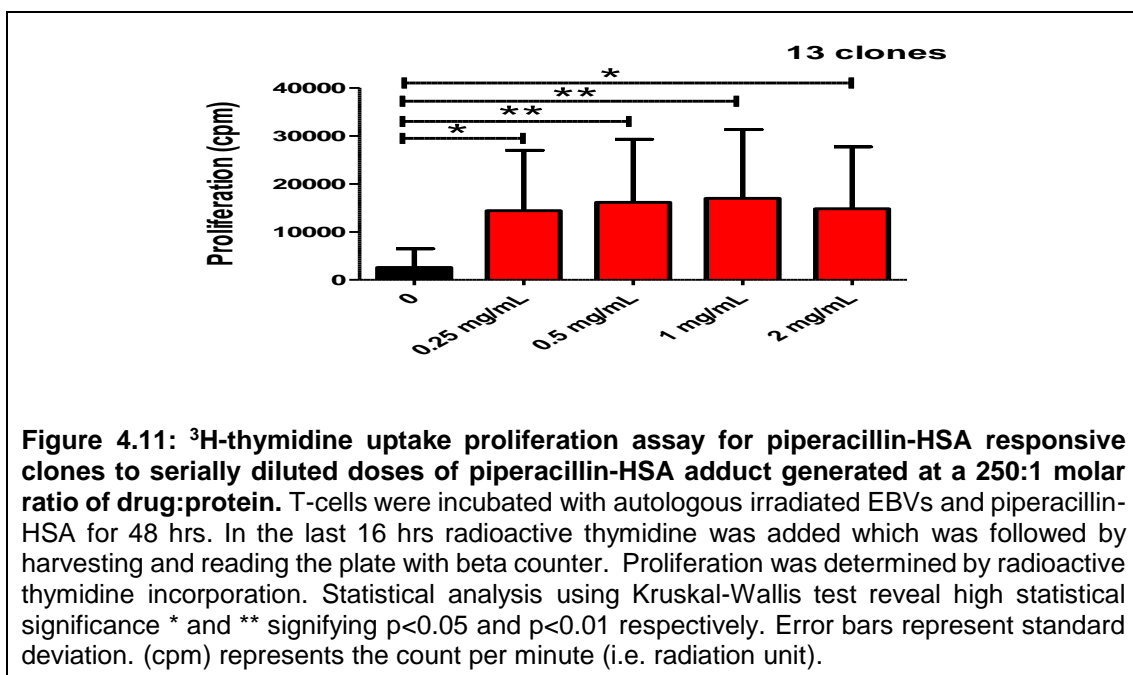
Table 4.2: Cross reactivity of piperacillin and piperacillin-HSA clones tested in Figure 4.8

	10 Clones primed to piperacillin-HSA conjugates	37 Clones primed to free piperacillin
Clones reactive to piperacillin-HSA adducts	10 (100%)	3 (8.1%)
Clones reactive to free piperacillin	5 (50%)	37 (100%)

4.4.5.4 Measurement of the dose-dependent proliferative response of piperacillin-HSA responsive clones

Thirteen clones were tested for their sensitivity to serially diluted doses of a piperacillin-HSA adduct generated with a 250:1 molar ratio of piperacillin-HSA using the ^3H -thymidine uptake proliferation assay.

A clear proliferative response to serially diluted doses of the HSA adduct was observed (**Figure 4.11**). Statistical significance was observed for all the selected doses ranging between (0.25-2 mg/mL) as analyzed by Kruskal-Wallis test.

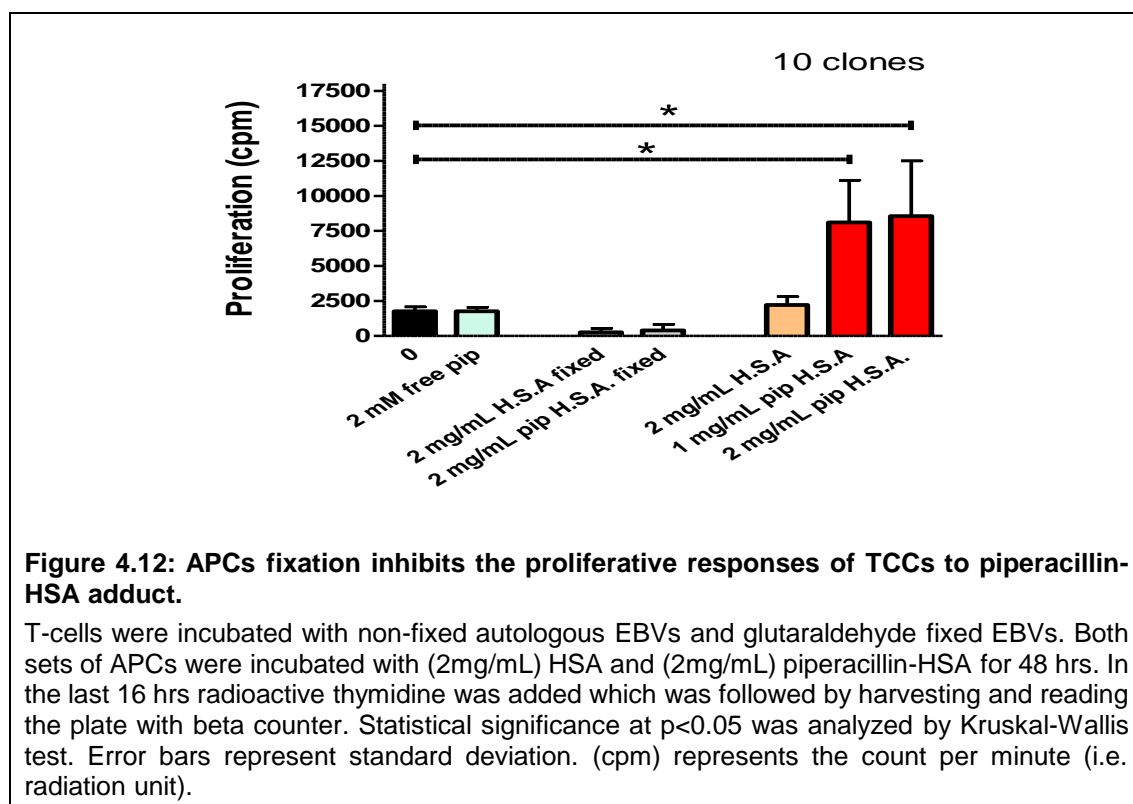


4.4.5.5 Proliferative response of T-cell clones with and without APC fixation

To confirm that the clones responsive against piperacillin-HSA were activated via a hapten mechanism involving protein processing, antigen presenting cells were fixed with glutaraldehyde. Fixation blocks the ability of the APC to process and present antigen (including drug-protein adducts), but not free drugs that bind

directly to MHC molecules expressed on antigen presenting cells (Yaseen et al., 2015; Zanni et al., 1999; Monshi, 2013). Fixation of antigen presenting cells prevented the activation of all clones with free piperacillin (El-Ghaiesh et al., 2012).

Ten clones were tested for their proliferative responses when challenged with piperacillin-HSA adducts, HSA and medium. These clones were incubated with irradiated autologous APCs and fixed irradiated autologous APCs.

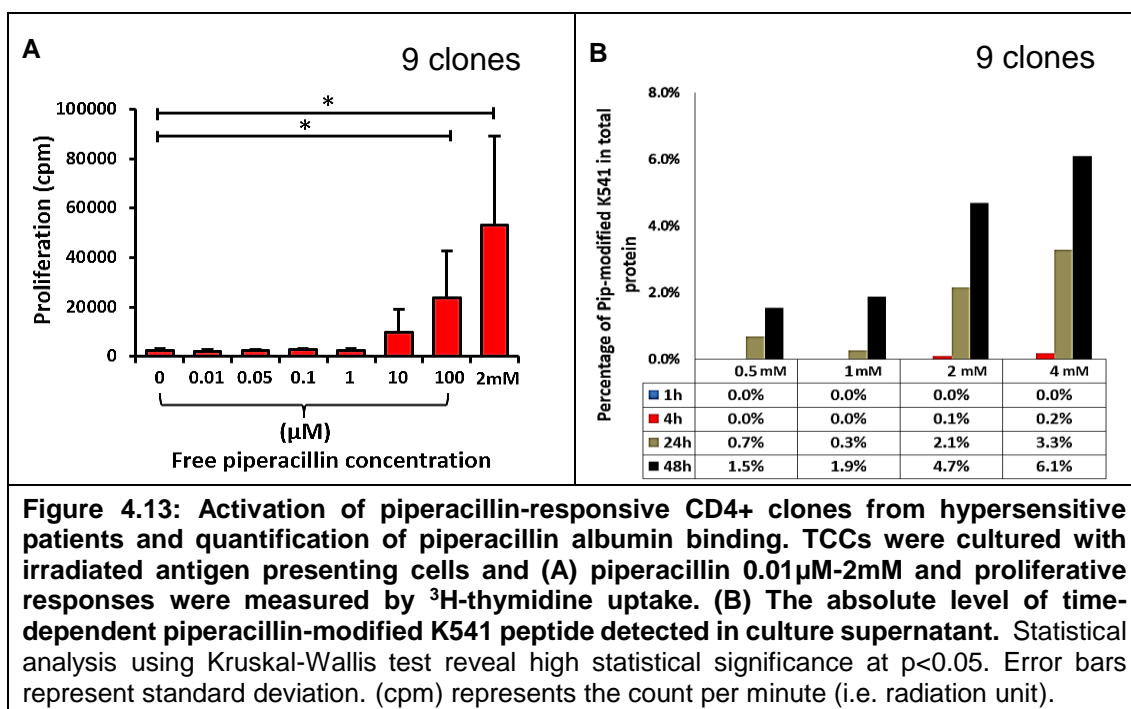


With irradiated APCs, the clones were activated with piperacillin-modified, but not unmodified HSA (**Figure 4.12**). Fixation of APCs completely inhibited the proliferative response to the protein adduct.

4.4.5.6 Quantification of the threshold level of piperacillin modified albumin required to trigger a T-cell response

Piperacillin modification of lysine residues at the lowest concentration of conjugate that activated T-cells (i.e. 0.25 mg/mL) (**Figure 4.11**) was calculated to be (2.9%) which was in a similar range to the level of modification detected in patients plasma (4%) (**Figure 4.5 B**).

In chapter 3, clones cultured with antigen presenting cells and titrated concentrations of piperacillin (0.125–2mM) were shown to be activated at the lowest concentration studied; thus, piperacillin concentration was reduced to establish a threshold for T-cell stimulation. A piperacillin-specific T-cell proliferative response was detected with 5 out of 9 clones at a concentration of 0.01mM. However, statistical significance was only reached with concentrations of 0.1mM and above (**Figure 4.13 A**). Coinciding with the T-cell response, piperacillin-modified K541 was detectable after 48h with 0.1mM piperacillin (approx. 1%). Increasing the concentration of piperacillin resulted in higher level of piperacillin hapten-modified Lys 541, with 4.7% modification detected at 2mM piperacillin. Moreover, hapten albumin binding was detected at earlier time-points (4-24h) with piperacillin concentrations ranging from 0.5-4mM (**Figure 4.13 B**).



4.4.5.7 Proliferative response of T-cells clones with APCs pulsed with free piperacillin

The level of piperacillin hapten adduct required for triggering T-cells was determined by pulsing antigen presenting cells with free piperacillin (2mM) for 1, 4, 24, and 48h prior to washing and exposure of the antigen presenting cells to free piperacillin responsive TCCs. Antigen-presenting cells pulsed with free piperacillin for 1 and 4 h did not stimulate a proliferative response and only low level of piperacillin-modified Lys541 were detected in the culture supernatants (**Figure 4.14**). In contrast, 24 and 48h pulsed antigen presenting cells stimulated all clones to proliferate and the strength of the induced response was equivalent to or stronger than that seen with the free drug. From this data, it can be estimated that HSA adducts with 2.8% of Lys541 modified by piperacillin act as antigens to activate T-cell proliferative responses.

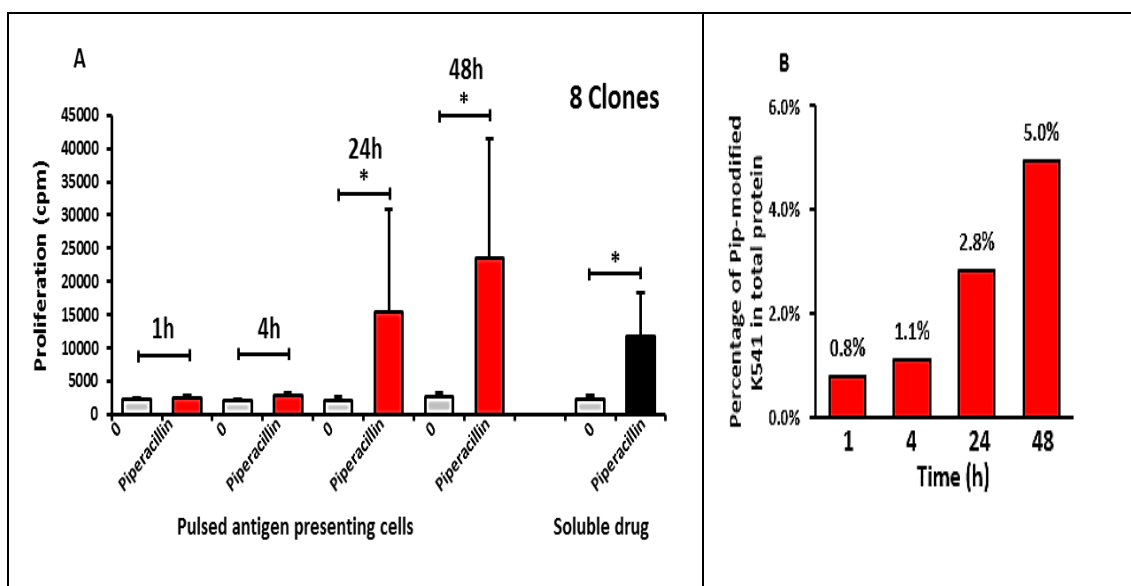
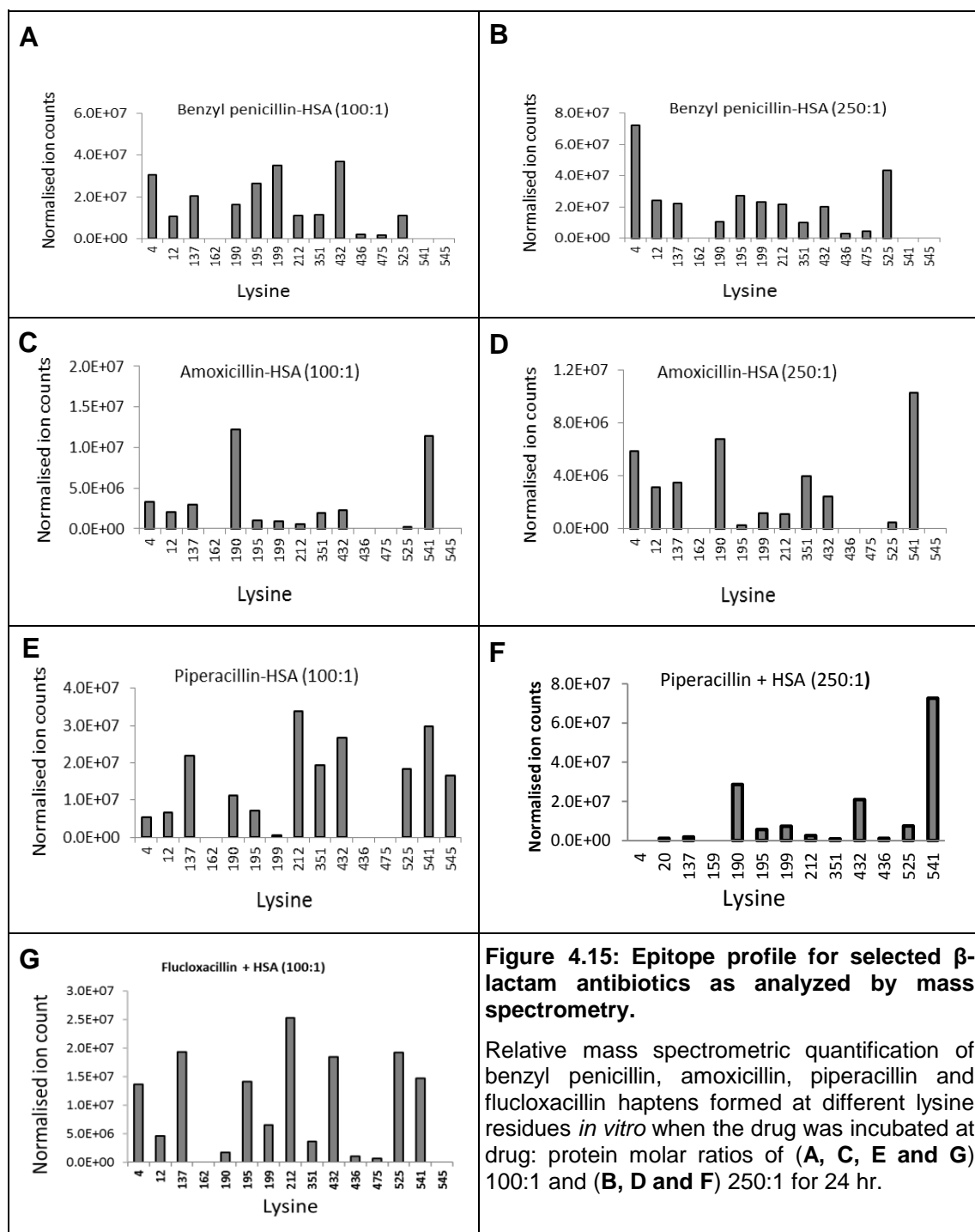


Figure 4.14: Time-dependent activation of CD4⁺ clones with free piperacillin-pulsed antigen presenting cells and quantification of piperacillin albumin binding. (A) TCCs (n=8) were cultured with antigen presenting cells pulsed with free piperacillin (2mM; 1-48h) and proliferative responses were measured by ³H-thymidine uptake. Antigen presenting cells were subjected to repeated washing with drug-free medium prior to exposure to clones. **(B)** The absolute level of time-dependent free piperacillin-modified K541 peptide detected in antigen presenting cell culture supernatant. Error bars represent standard deviation. (cpm) represents the count per minute (i.e. radiation unit).

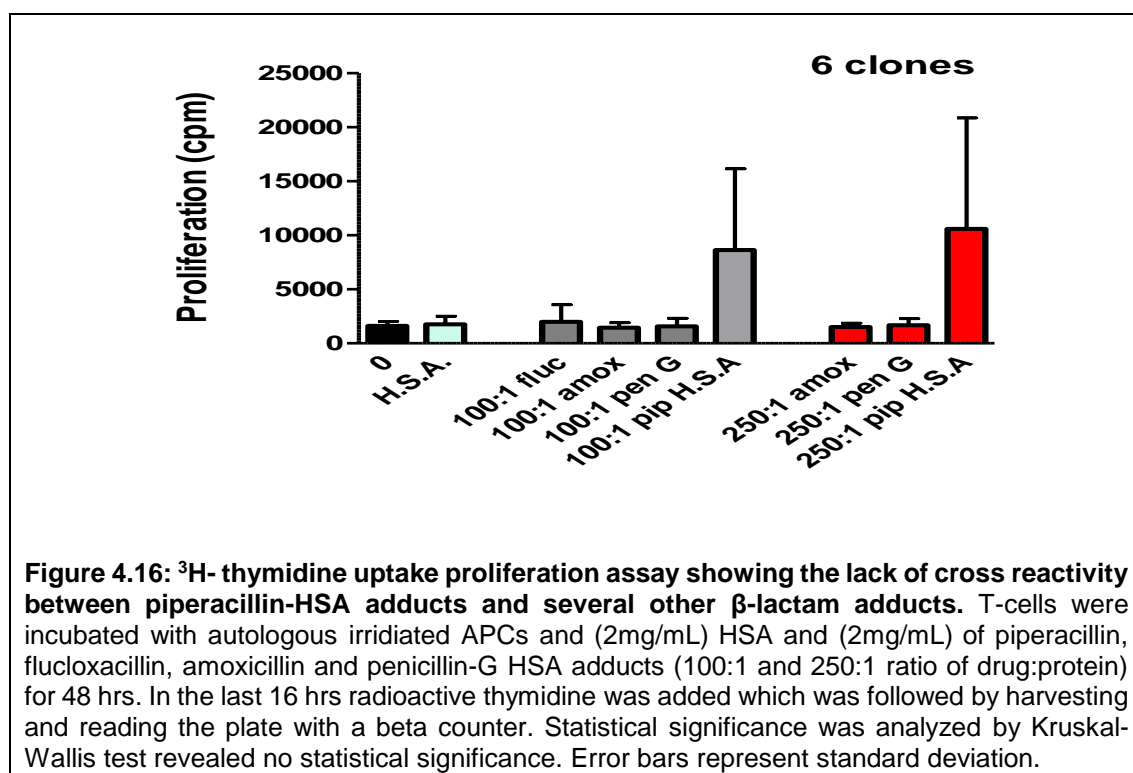
4.4.5.8 Cross reactivity between piperacillin-HSA adducts and HSA modified with other β -lactam antibiotics

Penicillin G, amoxicillin, piperacillin and flucloxacillin HSA adducts were generated by culturing the drugs with HSA at 100:1 and 250:1 ratios for 24hr (except for flucloxacillin which was prepared at only 100:1 molar ratio since the higher concentration damaged the tertiary structure of albumin). Epitope profiles showed that similar subsets of lysine residues were targeted by all four drugs; however, the relative level of binding differed at the individual sites of modification. Modification by penicillin G at Lys4, Lys137, Lys195, Lys199 and Lys432 resulted in strong MRM signals (**Figure 4.15 A and B**); however, Lys190 and Lys541 were primary targets for modification with amoxicillin (**C and D**), and Lys190, Lys212, Lys432, Lys525, and Lys541 were preferentially targeted by piperacillin (**E and**

F). For 100:1 flucloxacillin Lys4, Lys137, Lys195, Lys212 and Lys432 Lys525, and Lys541 were preferentially targeted (G).

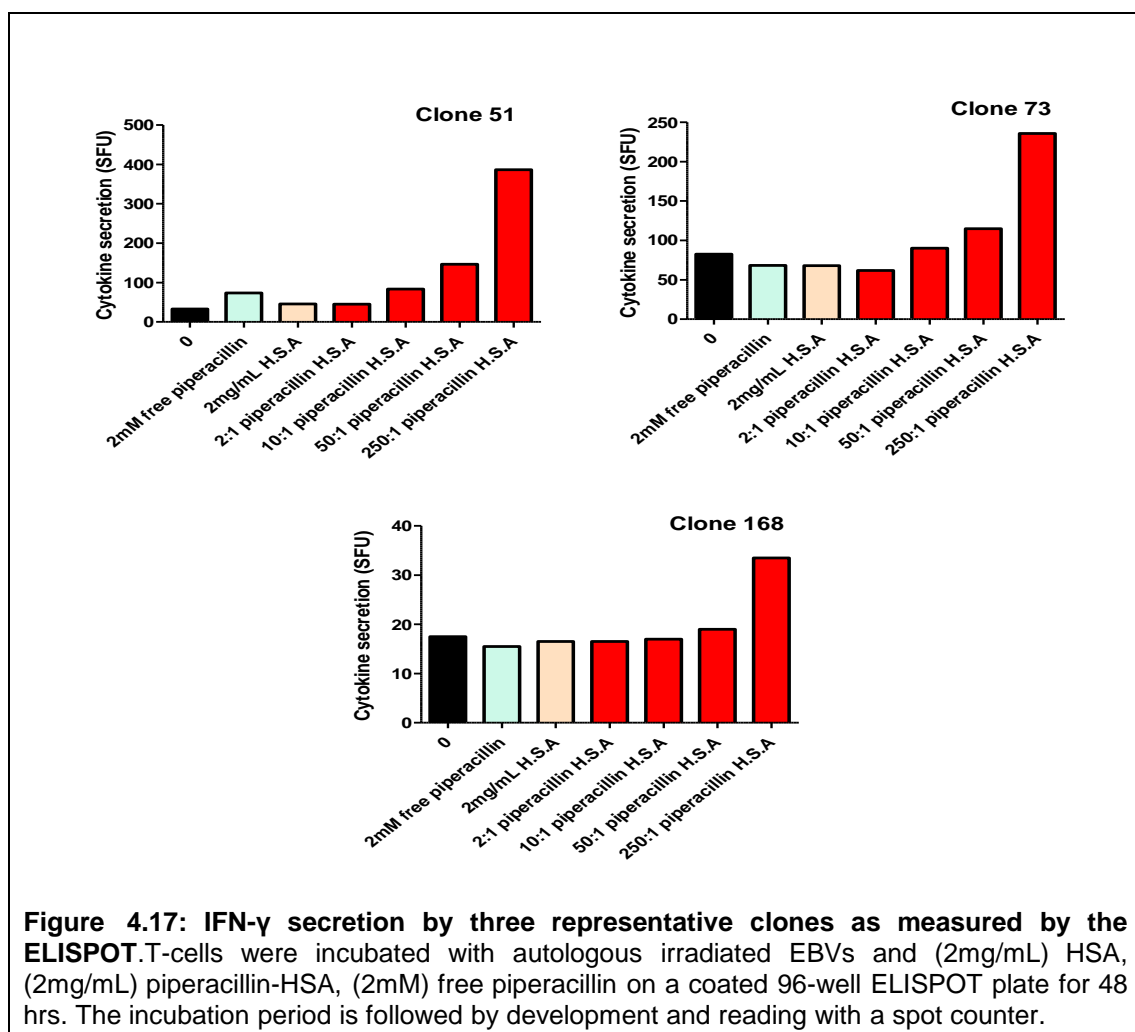


The proliferative response of piperacillin-HSA adduct responsive clones was tested with the other β -lactam adducts at a dose of 2mg/mL (**Figure 4.16**). Clones were stimulated to proliferate in the presence of the piperacillin-HSA adduct (slightly higher for the 250:1 in comparison to the 100:1 molar ratio), but not with HSA modified with the other β -lactams structures.



4.4.5.9 Quantitative and qualitative measurement of IFN- γ secretion profile of the T-cell clones by ELISPOT

Three clones were tested for secretion of IFN- γ using the ELISPOT technique when challenged with various molar ratios of the piperacillin-HSA adduct at the dose of (2mg/mL).



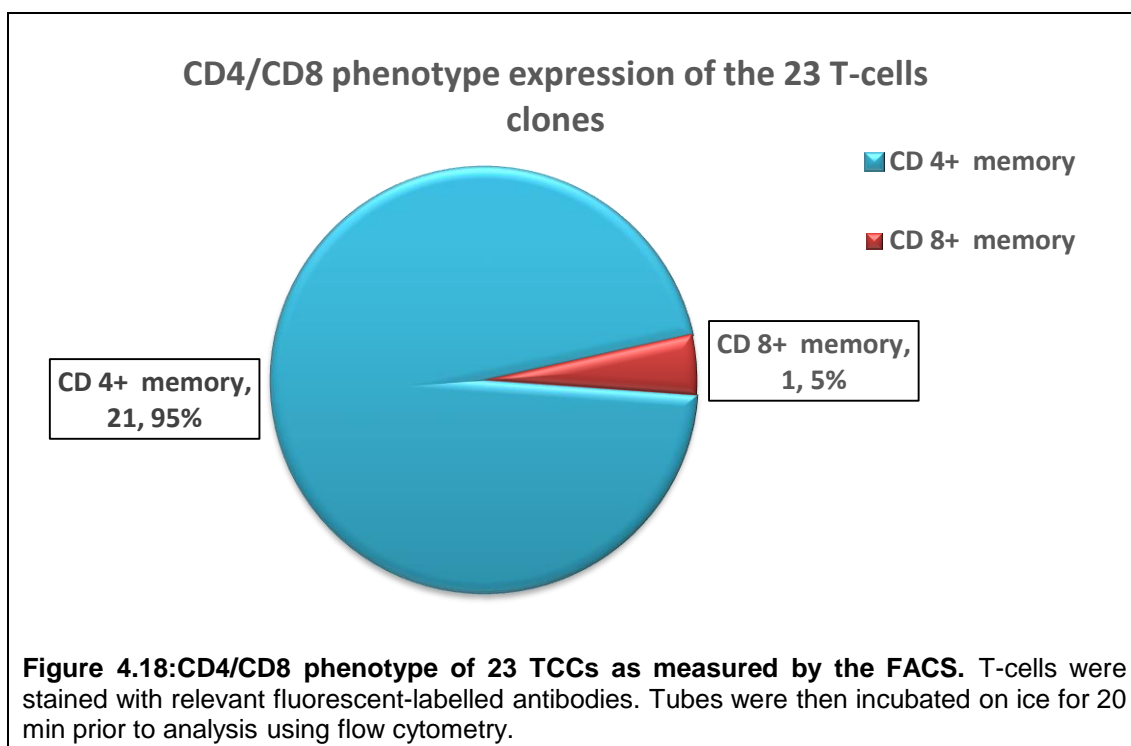
Clones were found to secrete IFN- γ in response to piperacillin-HSA adducts in a dose dependent manner with the highest response observed at a 250:1 molar ratio of drug:protein (**Figure 4.17**). Clones were stimulated to secrete only low levels of IFN- γ with free piperacillin.

4.4.5.10 Measurement of T cell receptors using the fluorescence-activated cell sorting (FACS):

- **CD4/CD8 phenotype, CD45RO expression (memory phenotype)**

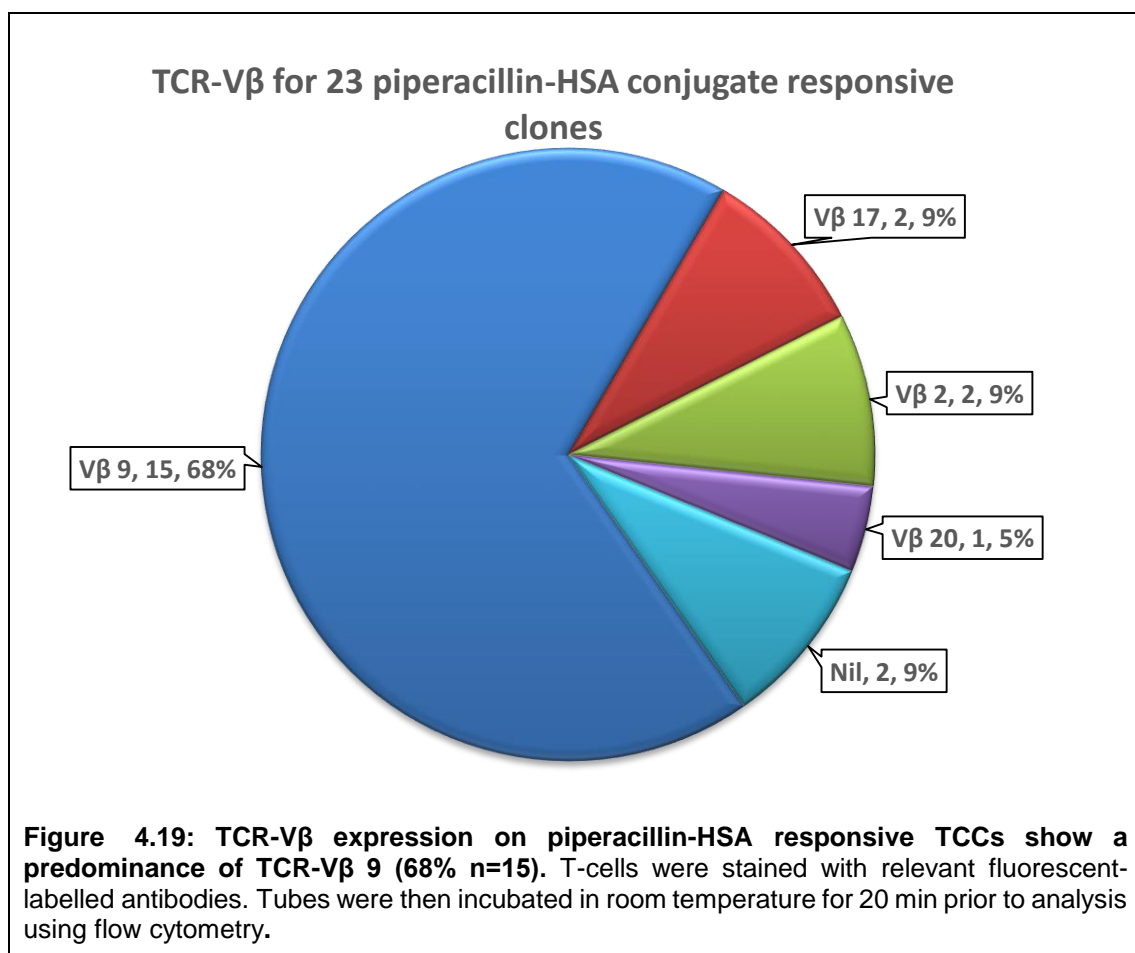
23 TCCs were analyzed using flow cytometry for expression of CD4, CD8, CD45RO and TCR-V β expression

Figure 4.18 shows that there is a predominance of the CD4 memory phenotype (95%, n=22) while only 1 clone expressed a CD8 memory phenotype constitutes (5%).



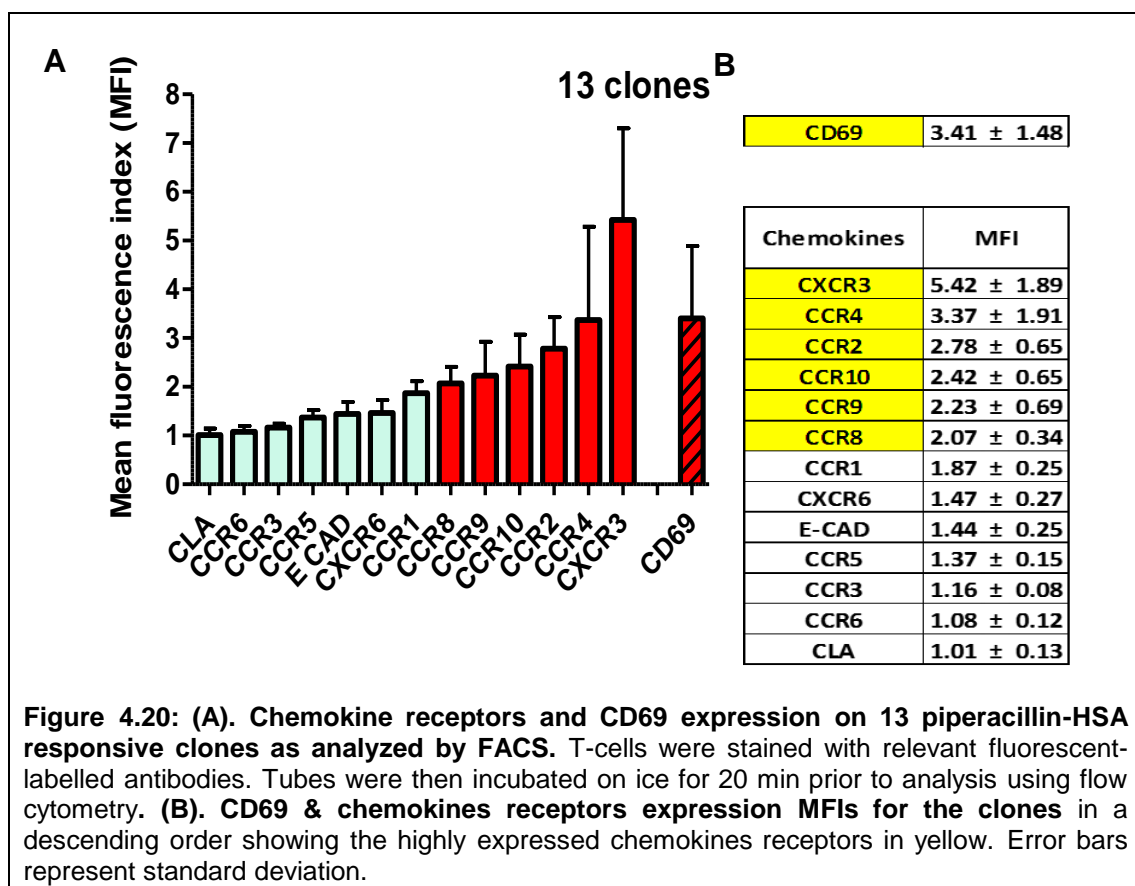
- **TCR-V β expression**

23 representative clones were analyzed for TCR-V β expression using flow cytometry. Clones expressed a restricted pattern of TCR-V β s. TCR-V β 9 was observed on 15 clones (68%), while other clones expressed the following TCR-V β s: TCR-V β 17 (n=2, 9%), 2 (n=2, 9%) and 20 (n=1, 5%). No TCR-V β expression was observed on 2 clones (9%) (**Figure 4.19**).



- Characterization of chemokine receptors and CD69 expression on piperacillin-HSA responsive T-cell clones**

13 TCCs were stained with fluorochrome labelled antibodies for chemokine receptors and then analyzed by flow cytometry. High expression of the following chemokine receptors was observed (mean fluorescence index greater than 2): CXCR3, CCR4, CCR2, CCR10, CCR9 and CCR8 in addition to the activation marker CD69 (**Figure 4.20**).



4.4.6 Characterisation of T-lymphocytes response from naive healthy volunteer to piperacillin-HSA adducts

In this part of the project, we tried to characterize the response of T-cells from the PBMCs of healthy normal (drug naive) donors primed to piperacillin HSA adducts.

Table 4.3: The volunteers' demographics. Three normal healthy volunteers were enrolled in this study.

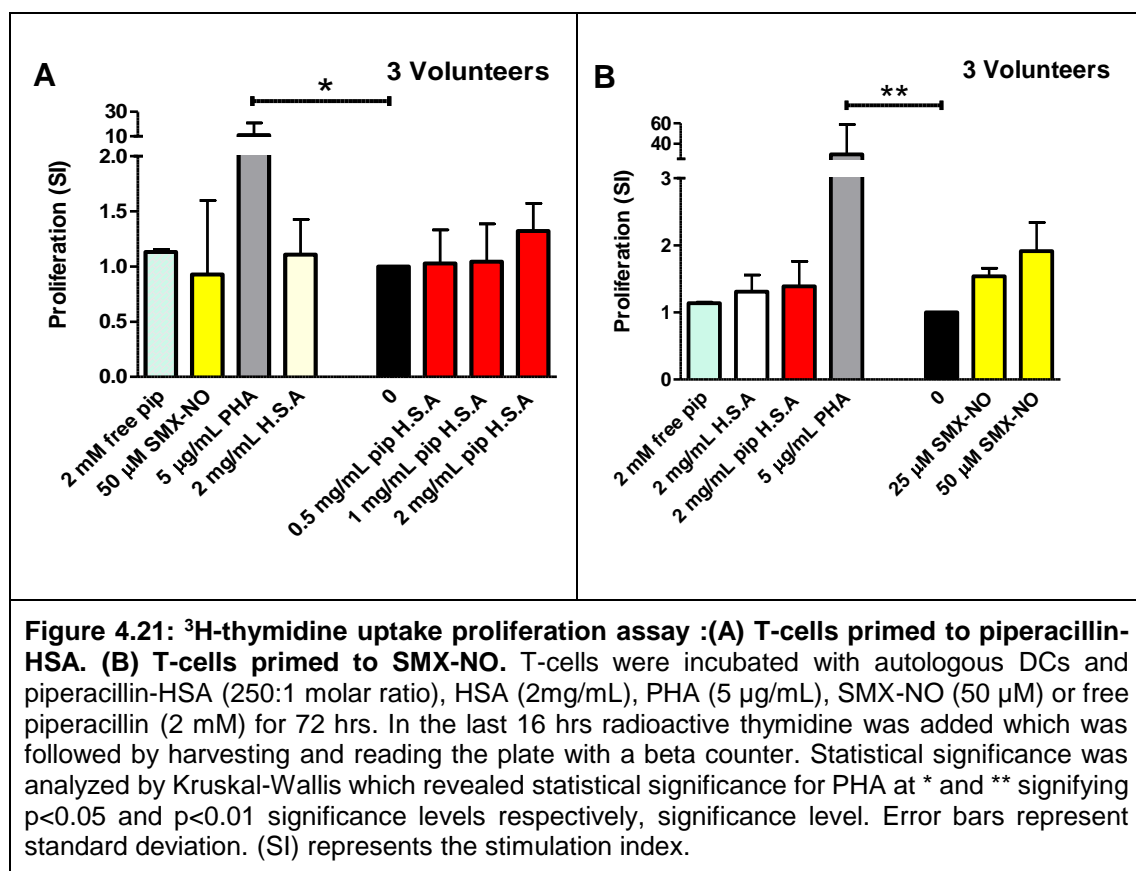
Donor ID	Age	Sex	History of allergies	Medical history
HVN-051	40	Male	Nil	Not relevant
HVN-066	36	Male	Nil	Not relevant
HVN-131	35	female	Nil	Not relevant

Blood was taken from three healthy volunteers (**Table 4.3**). Peripheral blood mononuclear cells (PBMCs) were separated from the blood. Naive T cells were

separated from the PBMCs. Dendritic cell-T-cell priming to piperacillin-HSA adducts (250:1 molar ratio) was conducted at the dose of (2mg/mL) in addition to another priming experiment using SMX-NO at (50 μ M) (to be used as an irrelevant drug antigen in the assays). T-cells were then harvested from the co-culture plates and tested for their specificity in proliferation and IFN- γ secretion assays.

4.4.6.1 ^3H -thymidine uptake proliferation assay

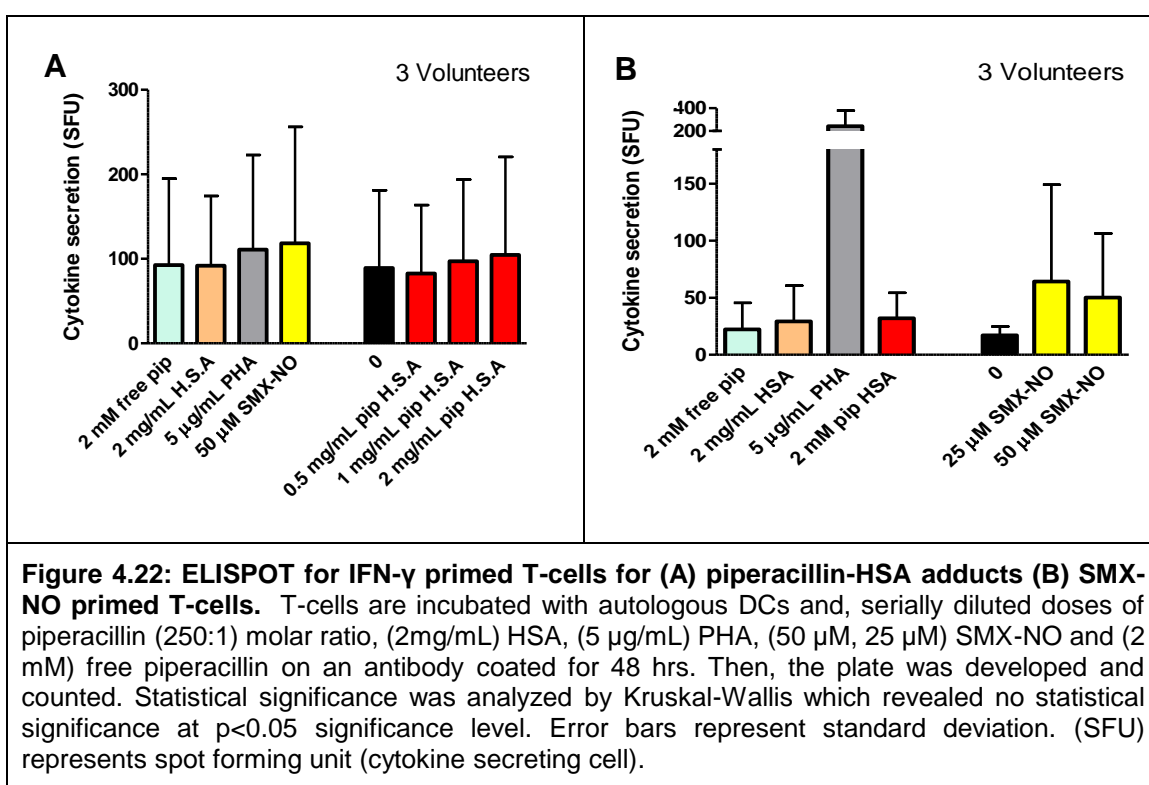
T-cells primed to piperacillin-HSA adducts did not proliferate when restimulated with a second batch of dendritic cells and either piperacillin HSA adduct (0.5-2mg/mL, 250:1 molar ratio) or SMX-NO. T-cells did proliferate vigorously in the presence of the positive control PHA (**Figure 4.21 A**).



In contrast, the SMX-NO primed T-cells (**Figure 4.21 B**) proliferated weakly in a dose-dependent manner with SMX-NO. The SMX-NO-responsive T-cells were

not stimulated to proliferate with piperacillin-modified HSA (2mg/mL). Similar data was obtained when IFN- γ ELISPOT was used as a readout for the drug antigen-specific T-cell response (**Figure 4.22 A and B**).

Clones were generated from the piperacillin-HSA primed cultures. However, following restimulation, none were found to be responsive to piperacillin-HSA (results not shown).



4.5 Discussion

Drug hypersensitivity reactions represent a significant clinical problem and an impediment to drug development. It is currently impossible to develop drugs with no immunological liability; furthermore, it is very difficult to predict which individuals will develop hypersensitivity when exposed to a therapeutic treatment regimen. This is because susceptibility is a function of the **chemistry of the drug**, **the genetic background of the patient** and **environmental factors** such as patient demographics, disease and concomitant medications. One of the predominant problems is the complexity of processes that deliver drug-derived antigens to the T-cell receptor. To activate CD4+ and/or CD8+ T-cells, the T-cell receptor must receive signals from an MHC-peptide-drug complex. *In vitro* analyses have revealed that certain drugs bind directly to the MHC peptide binding cleft and/or pre-bound peptides to activate T-cells (Yun et al., 2014; Adam et al., 2014; Illing et al., 2012; Ko et al., 2011; Ostrov et al., 2012; Chung et al., 2015).

However, the dominant pathway for drugs such as the β -lactam antibiotics (Monshi, 2013; Brander et al., 1995; Padovan et al., 1997; Yaseen et al., 2015) and sulphonamides (Elsheikh et al., 2011; Castrejon et al., 2010; Schnyder et al., 2000) involves the formation of a protein adduct with the drug hapten bound irreversibly to specific amino acid residues on non-MHC associated protein. The protein adduct is processed by antigen presenting cells liberating peptides that associate with MHC molecules to activate T-cells. The evolution of quantitative mass spectrometry methodology has allowed us to relate the level of drug hapten protein binding in patients to the activation of T-cells. In this study, analysis of hapten binding in patient's plasma and *in vitro* revealed that piperacillin binds to HSA at comparable levels. Thus, antigenic determinants with the potential to activate T-cells and cause tissue injury are formed in most patients exposed to the drug.

Our previous studies have demonstrated that piperacillin covalently modifies Lys residues at drug binding “Sudlow sites” on HSA in plasma (Whitaker et al., 2011). Moreover, piperacillin HSA adducts formed *in vitro* stimulate hypersensitive patient T-cells to proliferate and secrete cytokines (El-Ghaiesh et al., 2012). These data lead us to investigate whether the level of piperacillin HSA modification differs in individuals exposed to the same treatment regimen. Quantification of drug protein adducts has been always difficult. Mass spectrometric techniques have made it feasible to achieve relative quantification based on ion intensity of tryptic peptides. However, absolute quantification is only possible when mass spectrometric analysis is performed with a synthetic drug hapten peptide standard incorporating amino acid residues found in the native protein (Lange et al., 2008; Gallien et al., 2011).

Synthesis of β -lactam peptide adducts is extremely challenging; a specific synthetic strategy is required since direct penicilloylation of peptides occurs mainly on N-terminal amino group (unpublished data). Another major obstacle specific to piperacillin is its acid liability. Acid treatment of peptides either during chain elongation or during cleavage of the peptides from the support is a necessary component of peptide synthesis and such treatment causes degradation of piperacillin hapten and a partial degradation due to the acid susceptibility of thiazolidine ring. Consequently, provisions are required to avoid loss of the piperacillin group during peptide synthesis. We therefore developed a method utilizing piperacillin attached to the ϵ -amino group of lysine to produce a peptide containing Lys541 of HSA and the neighbouring amino acids (i.e., ATK(piperacillin)EQLK). The procedure utilized specific protecting groups for blocking reactive side chain groups in the peptide synthesis, which are readily removable under mild conditions without disrupting the piperacillin hapten. The synthetic peptide was chemically and physically indistinguishable from its endogenous counterpart in terms of retention time, ionization efficiency and fragmentation mechanism. Therefore, it was ideal to be used as a standard in targeted mass spectrometric quantification of piperacillin HSA binding.

4.5.1 CF patients plasma samples (piperacillin tolerant)

3.9% of Lys 541 in HSA isolated from plasma of patients exposed to piperacillin for 14 days was modified with piperacillin hapten. If one assumes that the albumin concentration in patient plasma is 40g/L and a typical plasma volume of approximately 2.4 litres, one can estimate that approximately 29.3 mg of piperacillin binds covalently to this single Lys residue in the average patient. Since up to 4 Lys are modified by piperacillin hapten, the overall body burden likely exceeds 80mg. The level of Lys541 binding ranged from 2.7-4.7%, indicating that there is a 2-fold difference in exposure to piperacillin-HSA adducts in patients exposed to the same treatment regimen. The analyses were conducted on three separate occasions to confirm that the difference does not originate from sample processing/analysis. Somewhat surprisingly, the hydrolyzed form of piperacillin hapten was the only moiety bound to HSA in patient plasma, which indicates that this form of the hapten almost certainly interacts with T-cell receptors expressed on antigen-specific T-cells.

4.5.2 Piperacillin hypersensitive patients derived T-cells clones

It is possible that TCCs cultured with free piperacillin are activated by the drug hapten bound covalently to protein carriers other than HSA. For this reason, synthetic β -lactam HSA adducts were generated to (1) confirm that piperacillin HSA adducts act as T-cell antigens (2) assess the relationship between the level of hapten binding and the T-cell response and (3) study T-cell reactivity with other β -lactam protein adducts.

Piperacillin HSA adducts were generated with 3.6-23.5% Lys541 modification by culturing piperacillin with HSA at ratios of 10:1-250:1. By analysing the adducts formed we have found that the percentage of peptide modifications in HSA are time and concentration dependent. This may explain the higher prevalence of piperacillin hypersensitivity reaction in CF patients since piperacillin is administered in high doses and for long durations to combat the more frequent, relentless bacterial infections. Such prolonged high doses would trigger the

generation of piperacillin-HSA adducts in sufficient levels for developing piperacillin hypersensitivity reactions.

The decision to use the (2mg/mL) of piperacillin-HSA adduct generated using a 250:1 drug:protein molar ratio for the generation of T-cell lines and for the testing of clones was based upon the following:

1. A previous study showed that this dose was the optimal for activating T-cells (El-Ghaiesh, 2011).
2. The dose response curve shows clearly that the conjugate generated at 250:1 piperacillin:HSA molar ratio has the highest percentage of lysine residues modified. Consequently, we assumed that this molar ratio would impose the strongest antigenic stimulus and therefore have the greatest chance of activating T-cells.

A panel of CD4+ clones displaying activity against piperacillin HSA adducts (responses against unmodified HSA were not observed) were expanded for the functional studies discussed below.

The response of all piperacillin HSA adduct-responsive clones was blocked by glutaraldehyde fixation indicating that the clones were activated via a hapten mechanism involving the generation of antigenic HSA-derived peptides. Furthermore, the response was restricted to the piperacillin hapten structure, as other β -lactam HSA adducts did not activate the T-cells. An increase in the level of piperacillin modification at Lys541 correlated with the strength of T-cell proliferative response (**Figure 4.14**).

After we generated free and conjugate specific clones, a range of experiments were conducted to explore the concentration and the time required for the activation to occur. In each experiment, we looked at the level of piperacillin-HSA binding as a marker for the level of binding that we see in the patients.

Free piperacillin clones, in general, are not responsive to HSA conjugates (**Figure 4.8 B**). However, the response of the clones was processing dependent. Thus, we assumed that they would respond to some form of protein adduct.

Thus, in experiments with free piperacillin responsive clones, modified HSA was used as a marker for the level of binding in culture after the activation of T-cells.

The data quantifying piperacillin protein adducts in plasma led us to measure the threshold level of HSA modification required to activate T-cells from hypersensitive patients. CD4+ TCCs were cultured with antigen presenting cells and titrated concentrations of piperacillin to ascertain the lowest drug concentration associated with a significant proliferative response. Supernatant was removed after 48h to quantify the level of piperacillin hapten HSA binding. Piperacillin-modified K541 was detectable at 0.1mM, the lowest concentration associated with the activation of T-cells. A dose-dependent increase in the level of modification was observed with piperacillin concentrations associated with a 20-fold increase in the proliferation of T-cells (i.e., 0.5-4mM). Moreover, Lys 541 binding was detected after only 4h with piperacillin concentrations of (2mM) and above. Importantly, equivalent levels of Lys541 modification were detected in patient plasma and *in vitro* with piperacillin concentrations that stimulated a T-cell proliferative response.

To estimate the absolute levels of HSA binding required for triggering T-cells, antigen-presenting cells were pulsed with piperacillin (2mM) for 1-48h prior to washing and exposure of clones to the pulsed cells. Supernatant was removed to quantify piperacillin hapten HSA binding at each time-point. Antigen-presenting cells pulsed with piperacillin for 1 and 4h did not stimulate T-cells and this coincided with low levels of Lys541 modification. In contrast, antigen presenting cells pulsed with piperacillin for 24h stimulated all clones to proliferate and the strength of the response was stronger to that seen with the free drug. At this time-point 2.8% of Lys541 was modified with piperacillin hapten.

On the other hand, piperacillin-HSA primed clones were firstly activated with an adduct generated at a drug:protein ratio of 10:1 (**Figure 4.8 A**) ; quantitative mass spectrometry revealed that 3.6% of Lys541 was modified with piperacillin hapten. Furthermore, from the dose response experiment based calculations (**Figure 4.11**) we found that piperacillin-HSA primed clones were activated by as lower as (2.87%) of piperacillin modified lysine residues.

In summary, both the free piperacillin and the piperacillin-HSA primed clones would respond to conjugates levels that are equivalent to that seen in patients plasma.

All together, these data imply that piperacillin protein conjugates play a pivotal role in piperacillin mediated hypersensitivity reactions.

Finding a proliferative response to piperacillin-HSA adducts without cross-reactivity to other β -lactams indicates that:

1. Penicilloyl core structure is not necessary alone to mediate specific antigenic signals to activate T-cells.
2. The piperacillin-HSA adduct antigenic determinants are highly specific.

Previous research has compared cross-reactivity of free piperacillin-specific TCCs with other β -lactam antibiotics. No cross reactivity was observed between free piperacillin and penicillin G and amoxicillin (El-Ghaiesh et al., 2012) which is similar to our current data with β -lactam protein adducts and confirms that the penicilloyl core structure is not the only determinant for drug-specific T-cell activation. To elaborate more, the previous study investigated the fine specificity of free piperacillin-specific responses using cefoperazone (3rd generation cephalosporin), which contains side chain closely related to piperacillin (**Figure 4.23**). The thiazolidine ring (of piperacillin) is replaced with 6 membered (dihydrothiazine) ring (for cefoperazone). Once more, there was no proliferative response to cefoperazone as compared with that of free piperacillin.

In summary, neither the penicilloyl core structure nor the side chains alone play an important role in mediating piperacillin specific responses. The missing ring in this conundrum is the specific piperacillin modified peptides which confer free piperacillin specific responses as shown in our experiment of cross-reactivity.

By comparing free piperacillin-specific clones to clones responsive (primed) against other β -lactam antibiotics such as flucloxacillin, we see a different cross-reactivity profile. Free flucloxacillin-specific clones are highly cross reactive. T-cell proliferative responses are also detected with piperacillin, amoxicillin, and penicillin G (Monshi et al 2013). Since the core β -lactams ring structure is the same in all the antibiotics, the study suggests that both the core penicilloyl structure and the MHC binding peptides provide the required energy to drive T-cell responses (**Figure 4.23**).

Another example of a drug hapten is SMX. SMX is an inactive drug (unable to form haptens) which undergoes metabolism to form SMX-NO, which forms conjugates with multiple cellular proteins and HSA. However, instead of modifying lysine residues it modifies cysteine and cysteine modified proteins have been shown to activate T-cells.

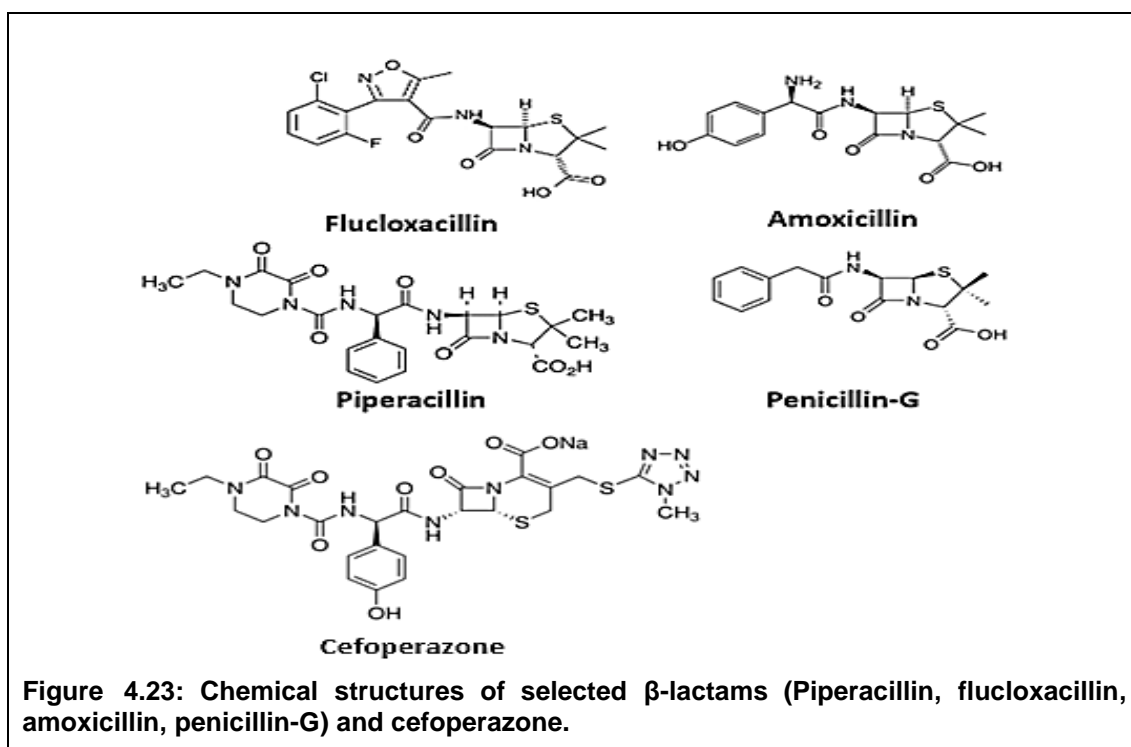


Figure 4.23: Chemical structures of selected β -lactams (Piperacillin, flucloxacillin, amoxicillin, penicillin-G) and cefoperazone.

Analysis of the clones using FACS technique showed a predominance of CD4+, which is consistent with that of free piperacillin responsive clones in the previous chapter.

FACS analysis also revealed that the clones show a restricted pattern of TCR-V β expression that is dominated by TCR-V β 9. This picture differs from that of the free piperacillin clones (previous chapter) which have shown that TCR-V β expression was diverse. This discrepancy may reflect different antigenic determinants, which are presented by APCs to mediate piperacillin specific activation of T-cells. However, comparing these results with that of DC-T-cell priming (bulks) of naïve healthy volunteers with free piperacillin (previous chapter) we can notice a striking similarity in terms of high expression of TCR-V β 9, which may propose a similar mechanism of T-cell activation and similar antigenic determinant. Chemokines expression profile is nearly identical to that expressed by the clones of free piperacillin (see previous chapter), however; differences in terms of SIs were noted. This profile can provide information on T cell subset i.e. these cells are a mixture of Th1 and Th2 cells.

The results of DC-T-cell priming to piperacillin-HSA conjugates show that the priming was unsuccessful. Further work is required to determine the reason for these negative results.

Collectively, our data reveal that the level of drug hapten protein binding in patients exposed to a therapeutic treatment regimen is sufficient to activate T-cells. Thus, it is interesting to speculate why most patients do not develop a drug antigen-specific T-cell response and clinical manifestations of hypersensitivity. It is possible that the covalent modification of protein *per se* is not sufficient to activate naïve T-cells. The consequence of adduct formation might be determined by multiple factors including co-stimulatory/inhibitory signalling and genetic factors such as HLA. The interplay between the previous factors altogether determines the net result of the piperacillin mediated immune response.

To further investigate the relationship between adduct formation and the allergic phenotype we have recently initiated a prospective investigation of piperacillin hypersensitivity. Bloods samples are being collected during repeated drug courses and when patients develop an adverse event to define the quantitative relationship between antigen formation and the clinical consequence of drug exposure.

Chapter Five

**Telaprevir &
Metabolite**

5 Characterization of the specificity and functionality of telaprevir specific T-cells isolated from naïve healthy volunteers

Telaprevir is the first antiviral protease inhibitor drug for the treatment of hepatitis C. However, its use was associated with high incidence of drug hypersensitivity reactions which were of obscure mechanism. Thus, we believed it was important to investigate why this drug is associated with such high incidence of frequently severe drug hypersensitivity reactions? Furthermore, telaprevir mediated drug hypersensitivity can be implemented as a model for other compounds in the same chemical class, i.e. protease inhibitors (e.g. boceprevir and semiprevir). We have chosen to investigate the metabolite VRT-127394 since it is a major metabolite and present in the blood of patients.

The best approach to explore such reactions is by investigating these reactions in telaprevir hypersensitive patients derived cells. However, we couldn't find such patient to recruit. Thus, we decided to work on naïve healthy volunteers derived T-cells.

We have addressed the following questions:

1. Is it possible to generate telaprevir & VRT-127394 metabolite-responsive TCCs from PBMCs of healthy naïve volunteers?
2. What is the phenotype of these clones (proliferation, cytokines secretion, TCR-V β and chemokine expression)?
3. Is there is a cross reactivity between telaprevir and VRT-127394 metabolite?
4. What is the possible mechanism of activation of these clones?

5.1 Aim of Study

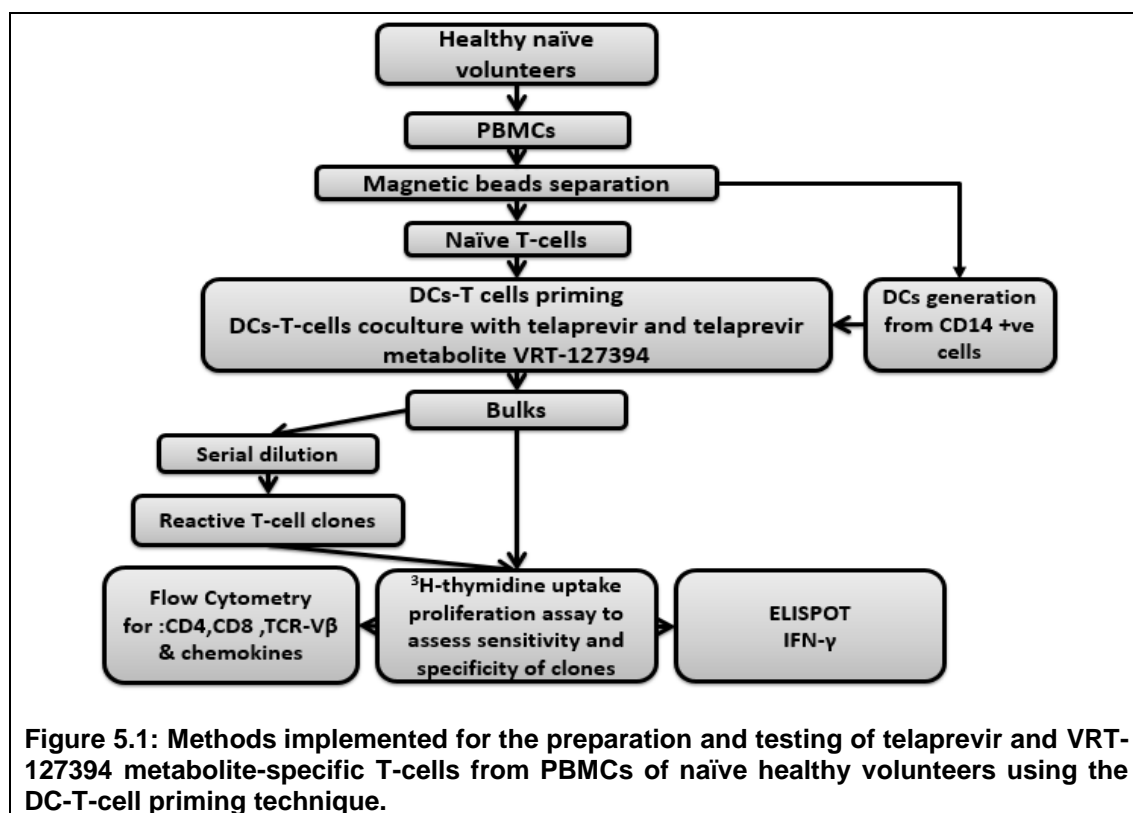
To identify telaprevir and/or telaprevir metabolite (VRT-127394) responsive T-cells in blood of healthy naïve donors and characterize their phenotype and function.

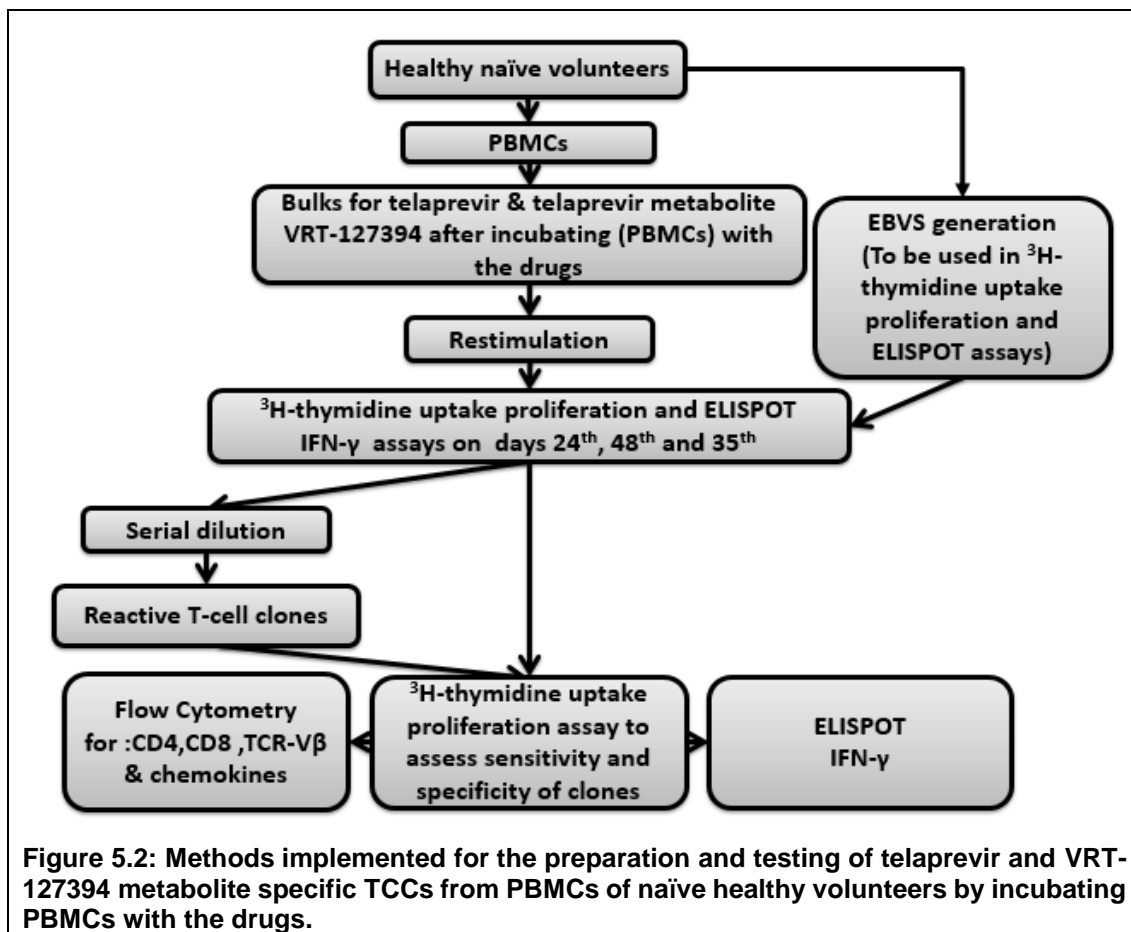
5.2 Materials & methods

Using PBMCs from naïve healthy volunteers, we tried to generate T-cells responsive against telaprevir and the metabolite VRT-127394 by implementing: Magnetic bead separation followed by **DC-T-cell priming** (

Figure 5.1). **PBMCs priming** by incubation with the drugs for 14 days followed by T-cell cloning using serial dilution (**Figure 5.2**).

After implementing these two methods, a battery of assays to characterize the drug-responsive TCCs were used as mentioned in **Figure 5.1** and **Figure 5.2**.





Note: Whenever the term **telaprevir-m** is mentioned in the results or discussion of this chapter, it denotes telaprevir metabolite VRT-127394 unless mentioned otherwise.

5.3 Results

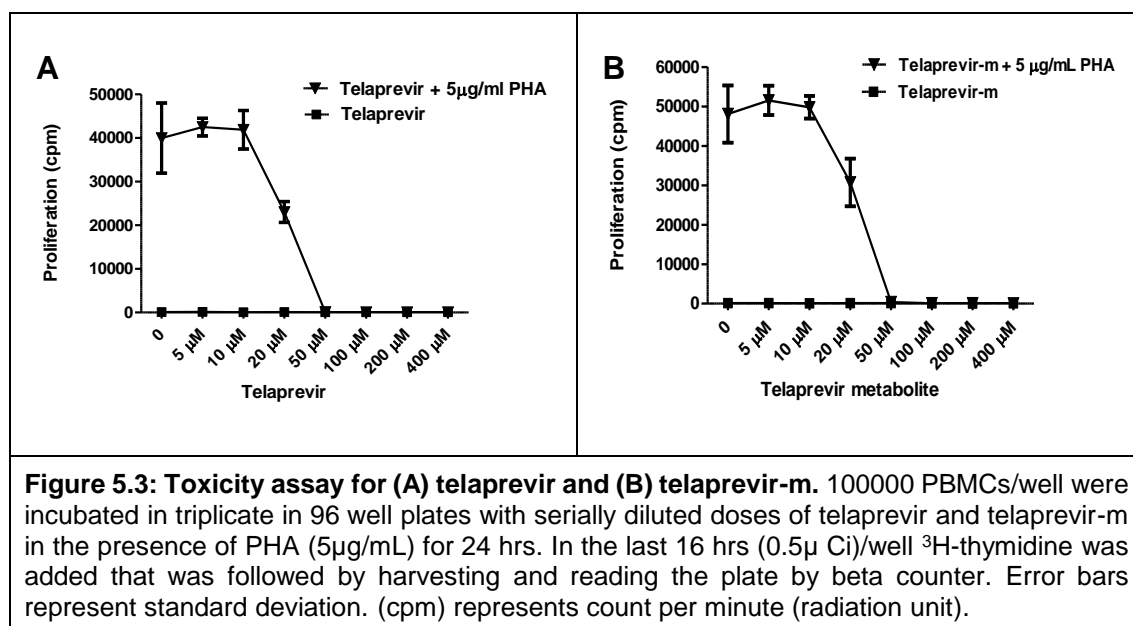
5.3.1 Choosing the volunteers

Three healthy drug-naïve volunteers were recruited in this study and their basic demographics are shown in **Table 5.1**.

Table 5.1: The healthy naïve donors demographics. Three healthy naïve volunteers were enrolled in this study to generate telaprevir and telaprevir-m specific T-cells using DCs-T-cell priming technique.				
Donor ID	Age	Sex	History of allergies	Medical history
V1	42	male	Nil	Not relevant
V2	37	male	Nil	Not relevant
V3	35	male	Nil	Not relevant

5.3.2 Identifying the drugs optimal dose for priming and T-cell assays (Toxicity assay)

PBMCs from one volunteer (V2) were incubated with serially diluted doses of telaprevir and telaprevir-m in the presence or absence of PHA (5µg/mL) for 24 hrs and ³H-thymidine was added in the last 16 hrs followed by harvesting and reading using beta counter. Fifty micromolar telaprevir and the telaprevir-m completely inhibited PHA-driven PBMCs proliferation (**Figure 5.3**). Thus, it was decided to use two concentrations, i.e. (20 µM) & (5 µM) for the generation of telaprevir and telaprevir-m specific T-cells.



5.3.3 Generation of telaprevir & telaprevir-m specific T-cells from healthy naïve volunteers using the DC-T-cell priming technique

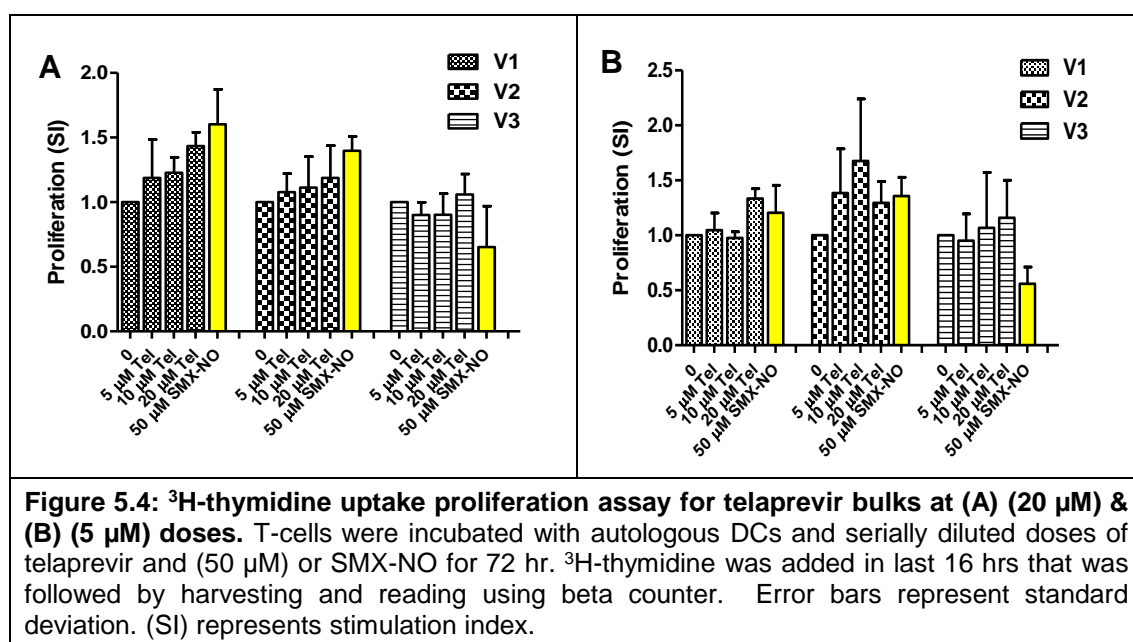
Using the DC-T-cell priming technique naïve T-cells were exposed to autologous dendritic cells and either telaprevir or telaprevir-m (20 μM & 5 μM). In the same experiments, SMX-NO was used as an irrelevant drug antigen. After 8 days, T-cells were tested for their drug-specificity using ³H-thymidine uptake proliferation and IFN-γ ELISPOT assays.

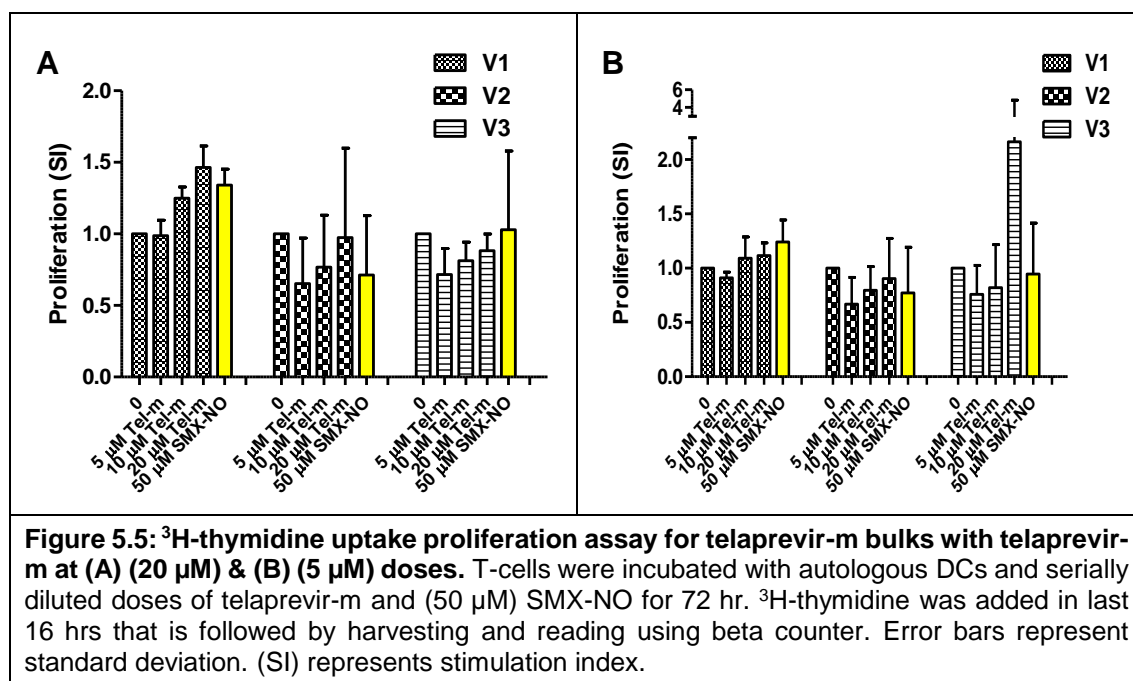
5.3.3.1 Quantitative and qualitative measurement of the telaprevir and telaprevir-m T-cell response using ³H-thymidine uptake assay

Telaprevir-specific T-cell proliferative responses were not observed when the telaprevir-primed T-cells were restimulated with dendritic cells and the relevant drug antigen. The SI was consistently lower than 1.5. (**Figure 5.4**). SMX-NO was

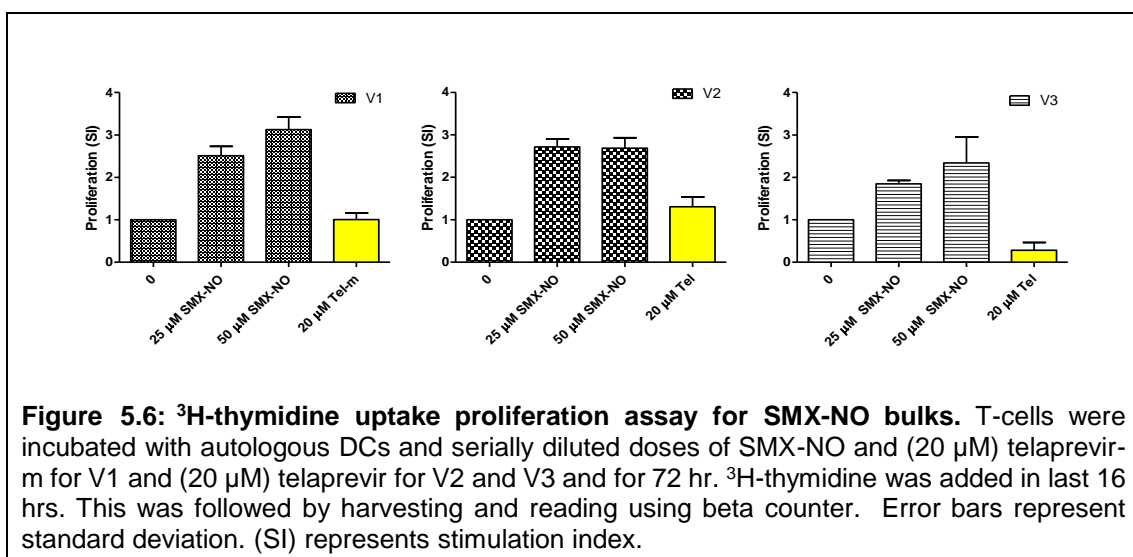
included as an irrelevant drug antigen and again the telaprevir primed T-cells were not stimulated to proliferate.

A similar pattern was observed with the telaprevir-m primed T-cells. Restimulation of the primed T-cells with telaprevir-m generally yielded SIs less than 1.5. However, a small increase in proliferation was observed with donor 3 at one drug concentration (**Figure 5.5**). For both sets of experiments there was no statistical significant response (as analyzed by Kruskal-Wallis test at $p < 0.05$ significance level).



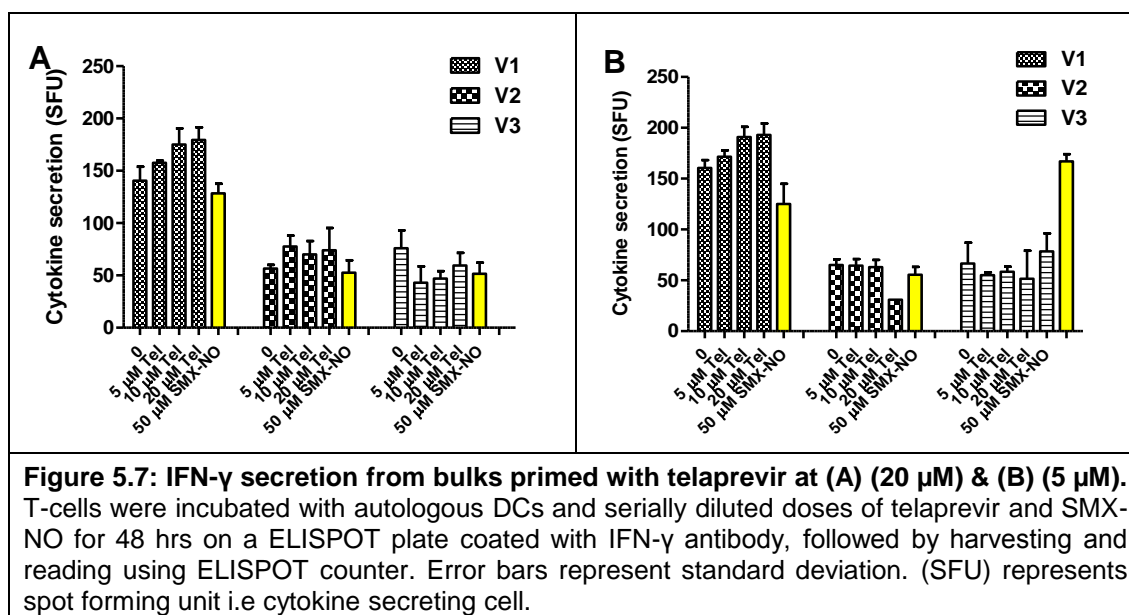


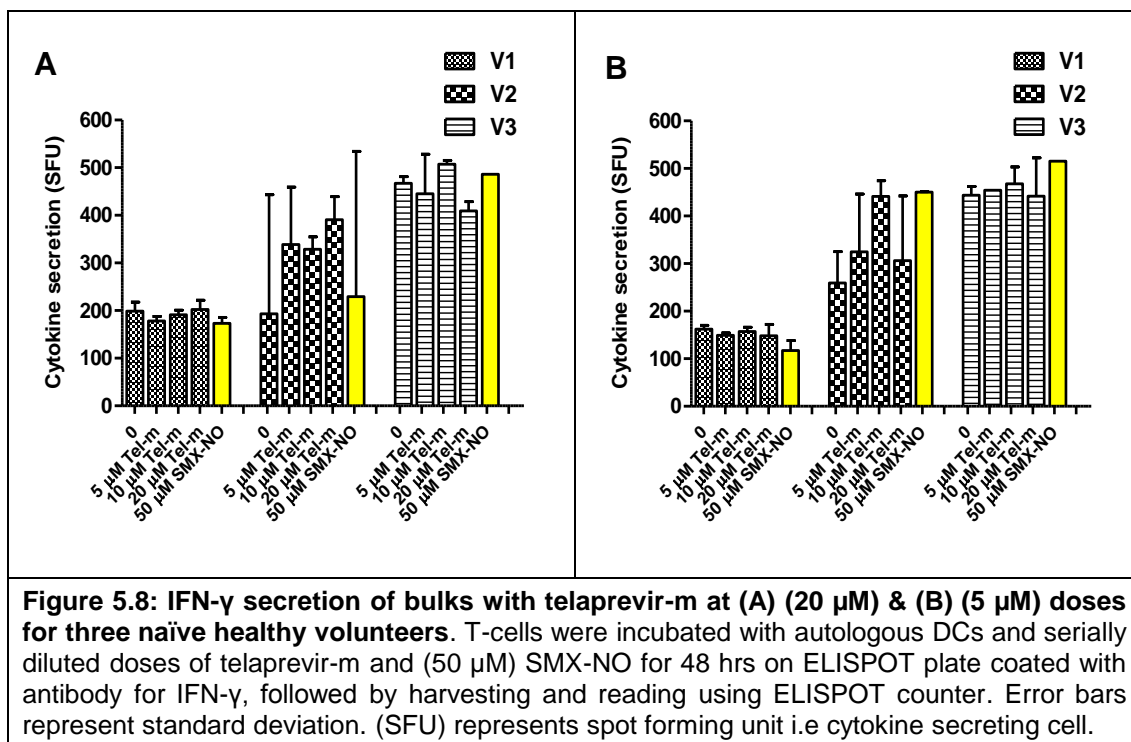
In contrast to telaprevir and telaprevir-m, SMX-NO successfully primed the naïve T-cells. Restimulation of the primed T-cells with SMX-NO resulted in a dose-dependent proliferative response in all three donors (**Figure 5.6**). Moreover, there was no cross reactivity with telaprevir or telaprevir-m.



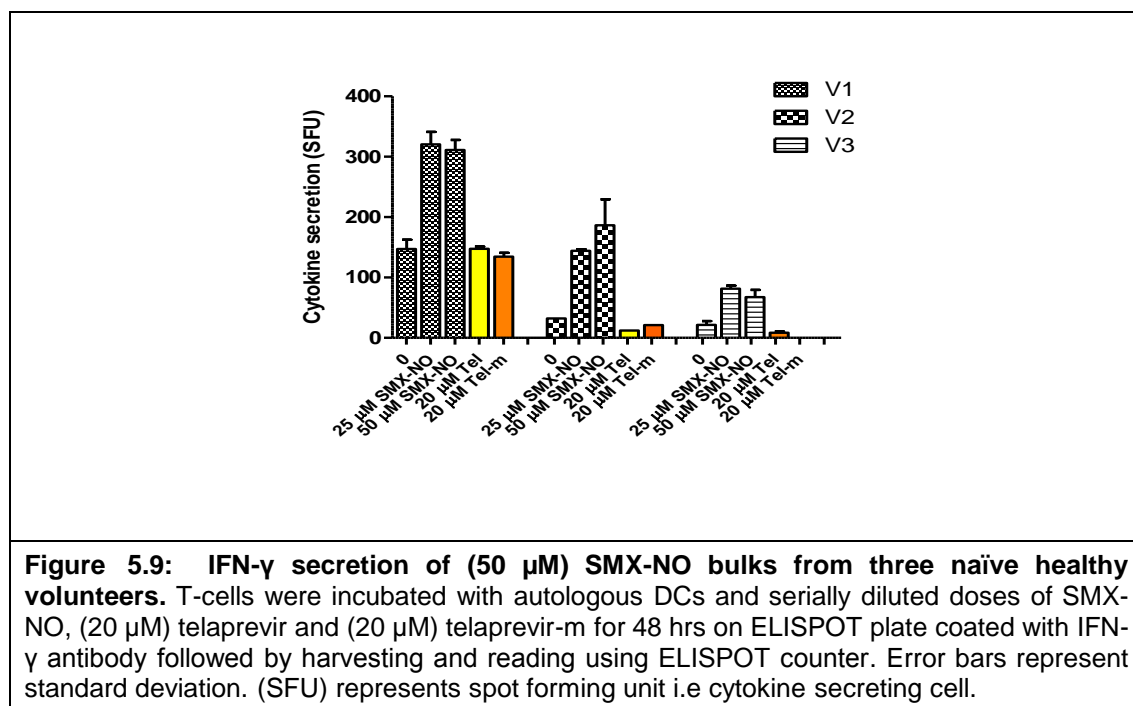
5.3.3.2 Quantitative and qualitative measurement of IFN- γ secretion of the bulks by ELISPOT

IFN- γ secretion from telaprevir, telaprevir-m and SMX-NO primed cells was measured using ELISPOT technique. In each experiment, the primed T-cells were restimulated with the relevant or irrelevant drug antigen prior to assessment of IFN- γ release. IFN- γ release from the telaprevir-primed T-cells was not detected (**Figure 5.7**). Similar results were obtained with T-cells primed against telaprevir-m (i.e. telaprevir-m-specific IFN- γ release was generally not detected (**Figure 5.8**)). A small increase in IFN- γ release was seen with donor 2 at both telaprevir-m priming concentrations; however, at lower priming concentration the level of cytokine released was the same as that seen with the irrelevant antigen SMX-NO.





In contrast, restimulation of SMX-NO-primed T-cells with SMX-NO resulted in the dose-dependent release of IFN- γ . No cross reactivity was observed with telaprevir or telaprevir-m (**Figure 5.9**).



5.3.3.3 Generation of telaprevir-m specific clones from PBMCs bulks

TCCs were generated from bulks of volunteer V1 using the serial dilution technique. 192 well growing clones were expanded. However, none of these clones were stimulated to proliferate in the presence of telaprevir or telaprevir-m.

5.3.4 Generation Telaprevir & telaprevir-m specific T-cells from healthy naïve volunteers using PBMCs priming technique

Three drug-naïve healthy volunteers were recruited, formally consented. Their basic demographics are summarized in **Table 3.4**

Table 5.2: The volunteers' demographics. Three naïve healthy volunteers were enrolled in this study to generate telaprevir and telaprevir-m specific T-cells using PBMCs priming technique.				
Donor ID	Age	Sex	History of allergies	Medical history
V4	35	female	Nil	Not relevant
V5	24	female	+ve for penicillin	Not relevant
V6	36	Male	Nil	Not relevant

5.3.4.1 Quantitative and qualitative measurement of the telaprevir, telaprevir-m bulks reactivity using ^3H -thymidine uptake assay

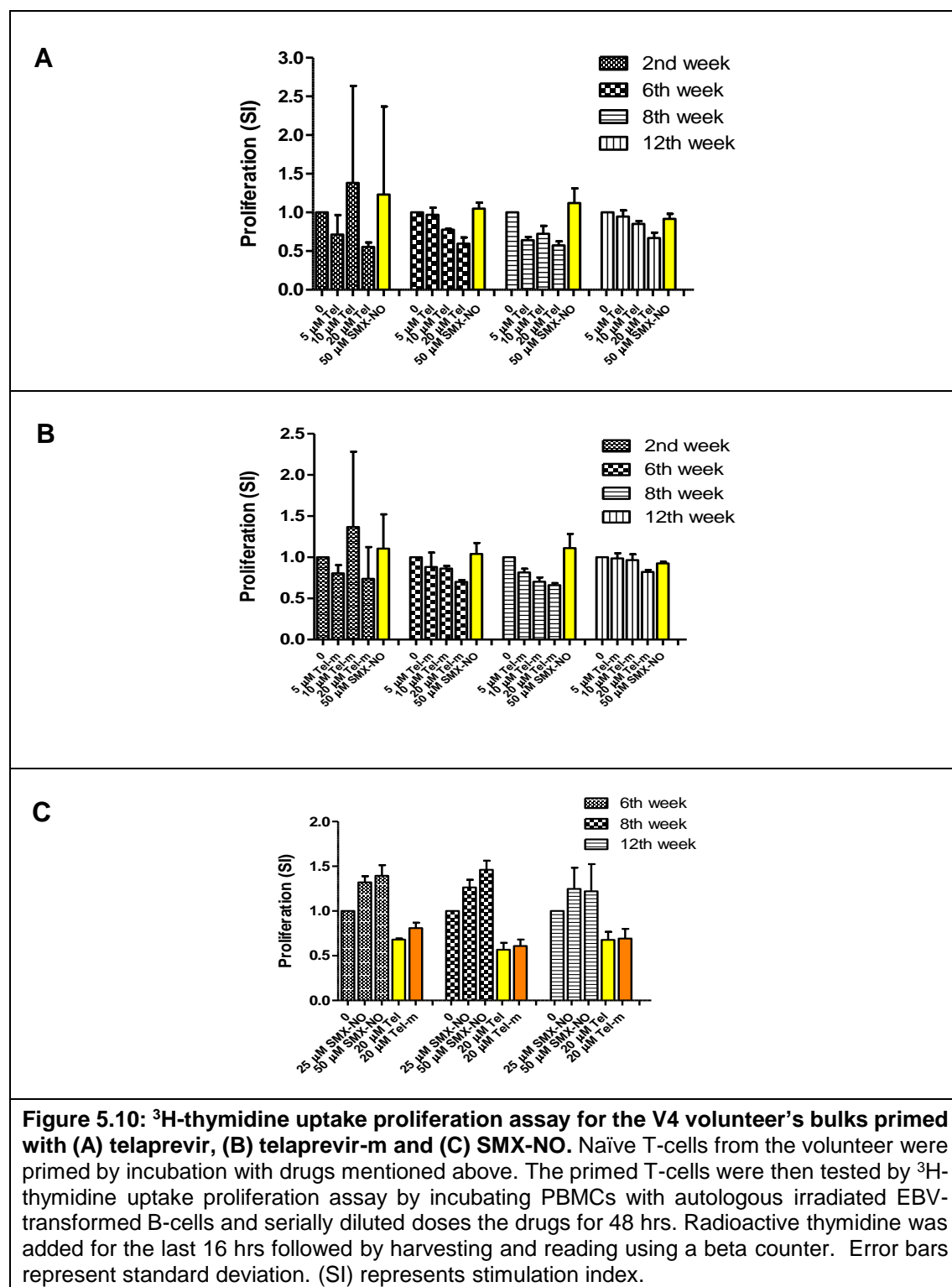
PBMCs from three volunteers were incubated with telaprevir (20 μM), telaprevir-m (20 μM) and SMX-NO (50 μM) for different time intervals. After 2-3 weeks, T-cells assays (^3H -thymidine uptake proliferation assay and IFN- γ ELISPOT) were carried out to test for antigen specificity. T-cell lines were then restimulated every 2-4 weeks with irradiated autologous PBMCs and the relevant drug antigen. The timing of testing is shown in Table 5.3.

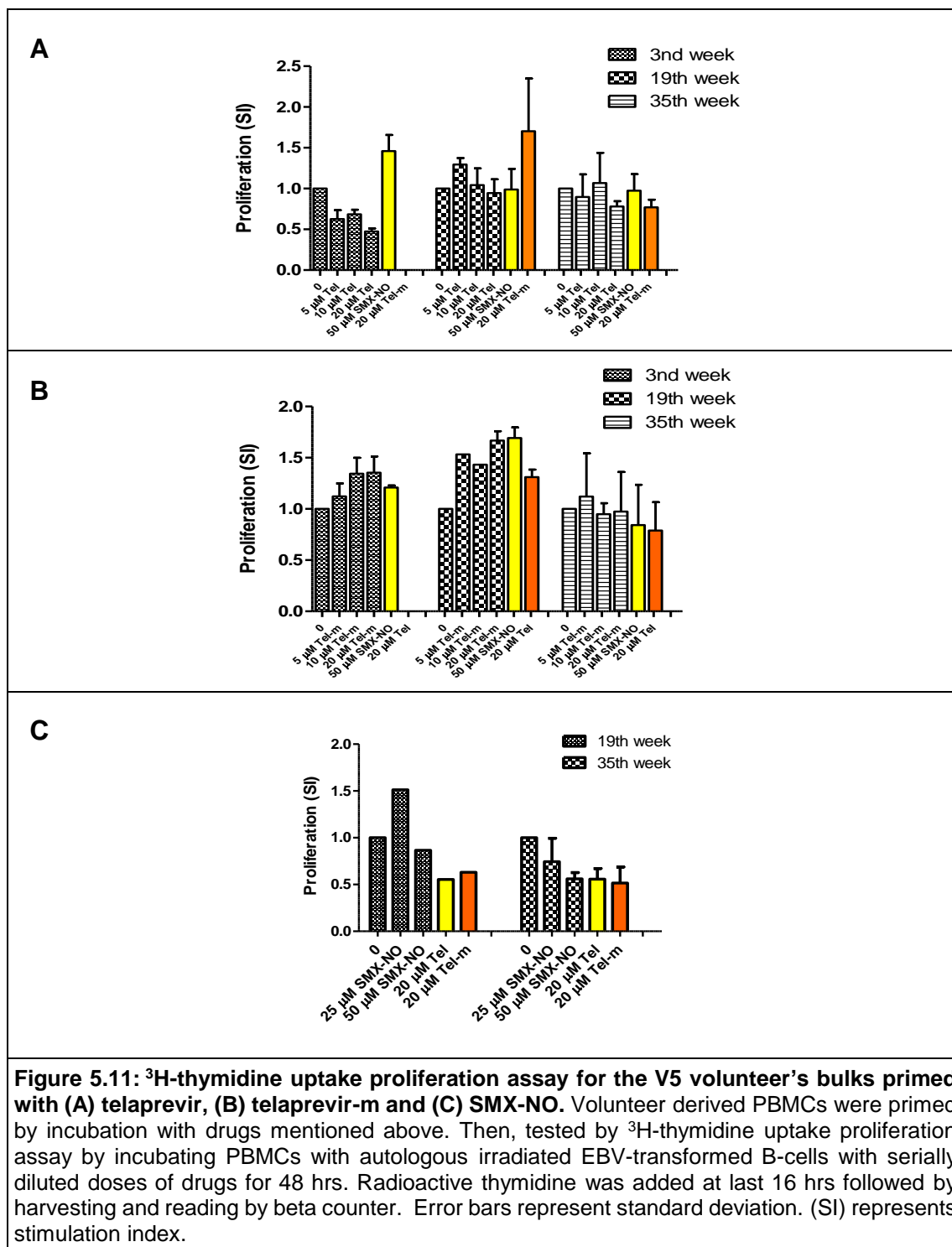
Table 5.3: Timing of ^3H -thymidine uptake proliferation assay and IFN- γ ELISPOT performed on bulks of the three volunteers. PBMCs were counted to make up (4-6x10⁶ cells/mL) in medium. Then, aliquoted into 24-well plate as (1mL/well) to make three wells. Telaprevir, telaprevir-m and SMX-NO were added (1mL/well) to make the final concentration of the drugs as follows (20 μM telaprevir), (20 μM telaprevir-m) and (50 μM SMX-NO). The plate was cultured at 37°C and 5% CO₂ for 5 days. From day 5, the wells were fed by withdrawing (600 μL) and adding (660 μL) of medium containing (2 μL /mL) IL-2 every 2-3 days. T-cells assays responses were carried out at the dates of highlighted boxes in the table below which include ^3H -thymidine proliferation assay & IFN- γ ELISPOT secretion. Highlighted squares refer to dates of both (proliferation & ELISPOT) as follows: (orange): telaprevir, (red): telaprevir-m and (black): SMX-NO.

Volunteers ID	Drugs	Weeks																																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36		
V4	Tel		■				■		■				■																										
	Tel-m		■				■		■				■																										
	SMX-NO						■		■				■																										
V5	Tel			■																	■																	■	
	Tel-m			■																	■																■		
	SMX-NO																				■																■		
V6	Tel		■					■																■															
	Tel-m		■					■																■															
	SMX-NO		■					■																■															

The proliferative response of T-cells stimulated with the drugs for each volunteer bulks are shown in **Figure 5.10**, **Figure 5.11** and **Figure 5.11**. In general, telaprevir and telaprevir-m did not stimulate cells to proliferate. SIs were lower

than 1.5 with no clear dose-dependency observed. In contrast, SMX-NO stimulated a stronger dose-dependent proliferative response when compared with telaprevir and telaprevir-m primed T-cells; however, the SIs were lower than 1.5. There was no statistical significance as measured by Kruskal-Wallis test at $p < 0.05$ significance level in all tests.





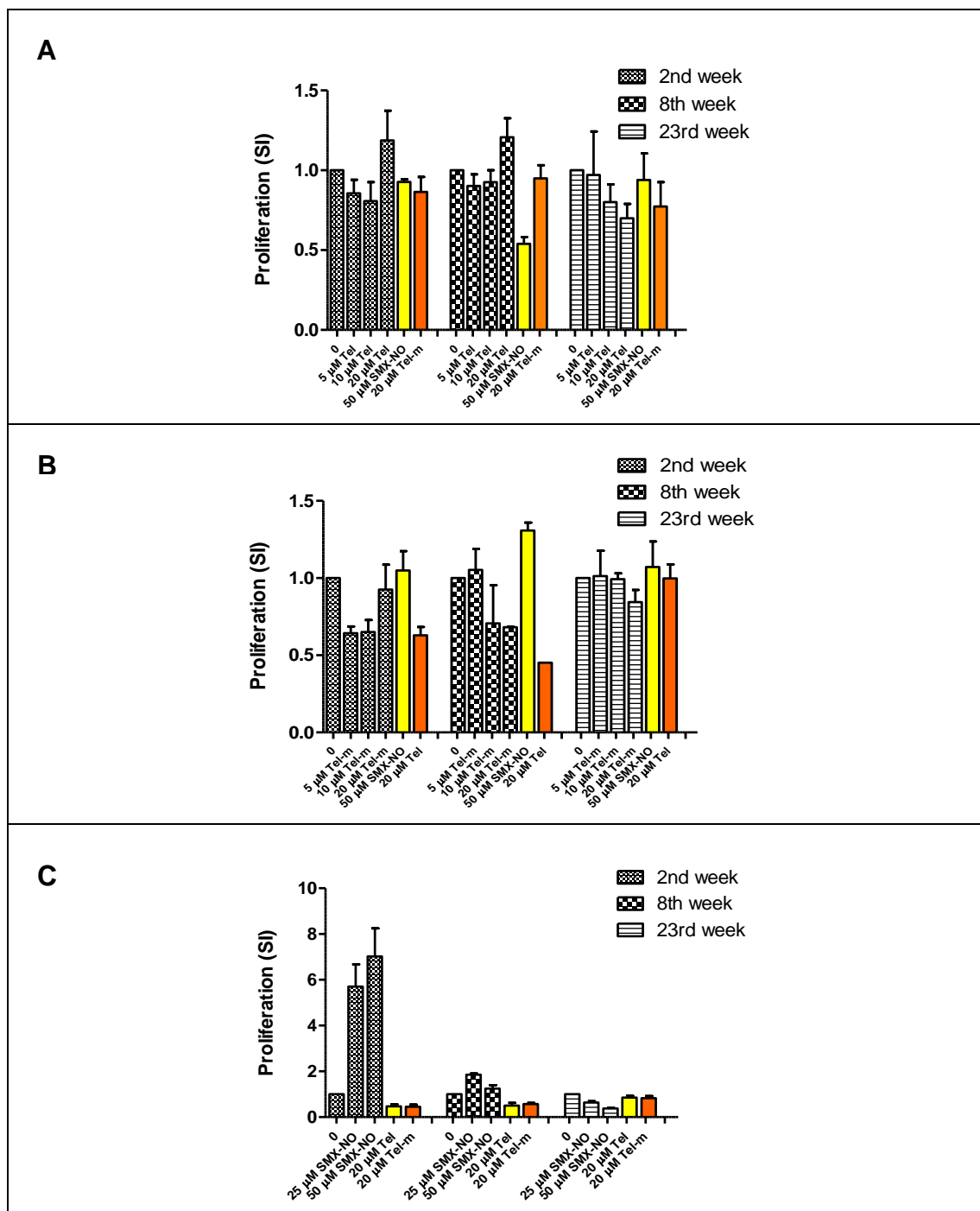


Figure 5.12: ^3H -thymidine uptake proliferation assay for the V6 volunteer's bulks primed with (A) telaprevir, (B) telaprevir-m and (C) SMX-NO. Volunteer derived PBMCs were primed by incubation with drugs mentioned above. Then, tested by ^3H -thymidine uptake proliferation assay by incubating PBMCs with autologous irradiated EBV-transformed B-cells with serially diluted doses of drugs for 48 hrs. Radioactive thymidine was added at last 16 hrs followed by harvesting and reading by beta counter. Error bars represent standard deviation. (SI) represents stimulation index.

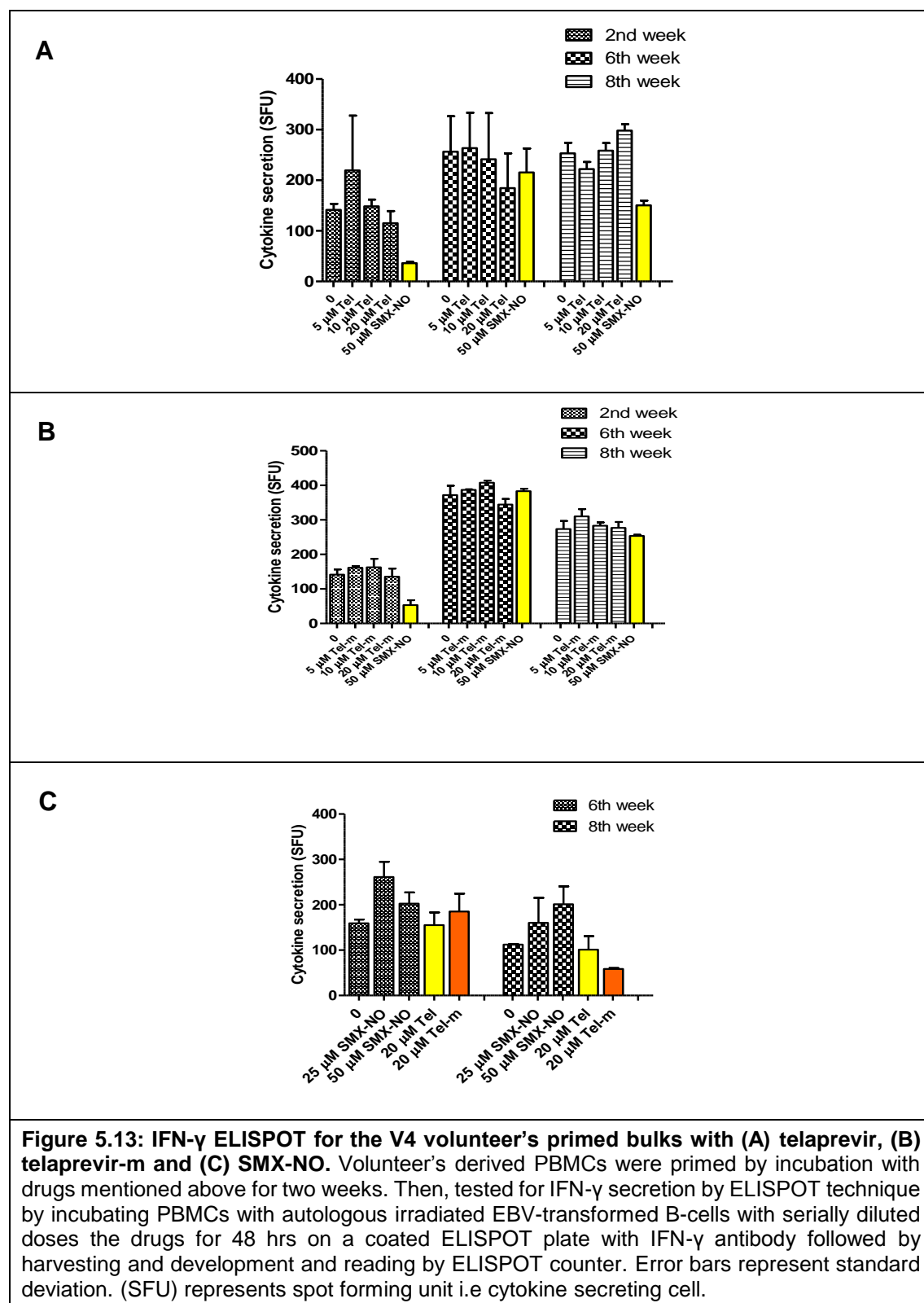
5.3.4.2 Quantitative and qualitative measurement of IFN- γ secretion from telaprevir and telaprevir-m-primed naïve T-cells

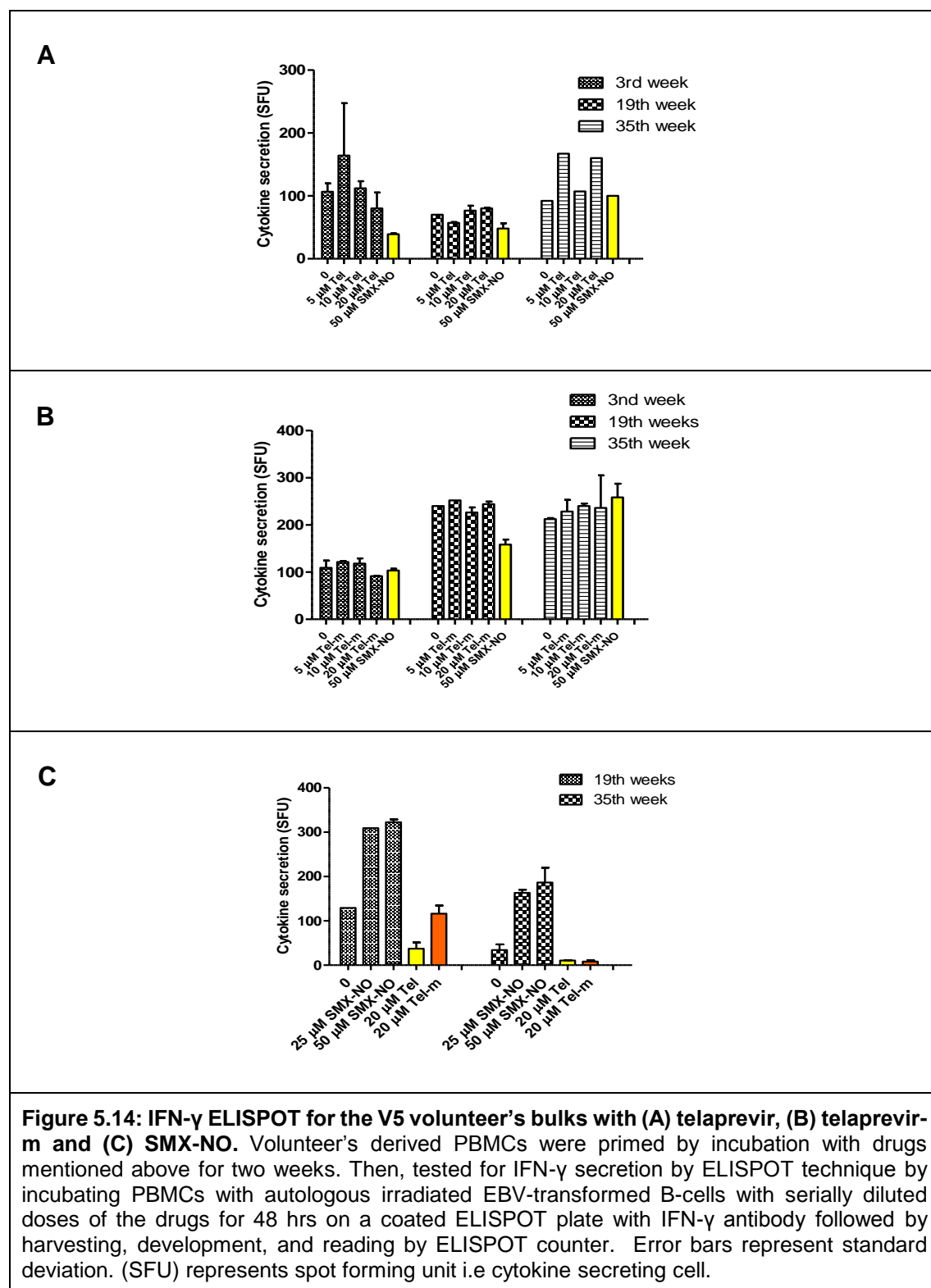
Naive T-cells primed against telaprevir, telaprevir-m and SMX-NO were tested for IFN- γ secretion using ELISPOT following restimulation with dendritic cells and relevant or irrelevant drug antigen.

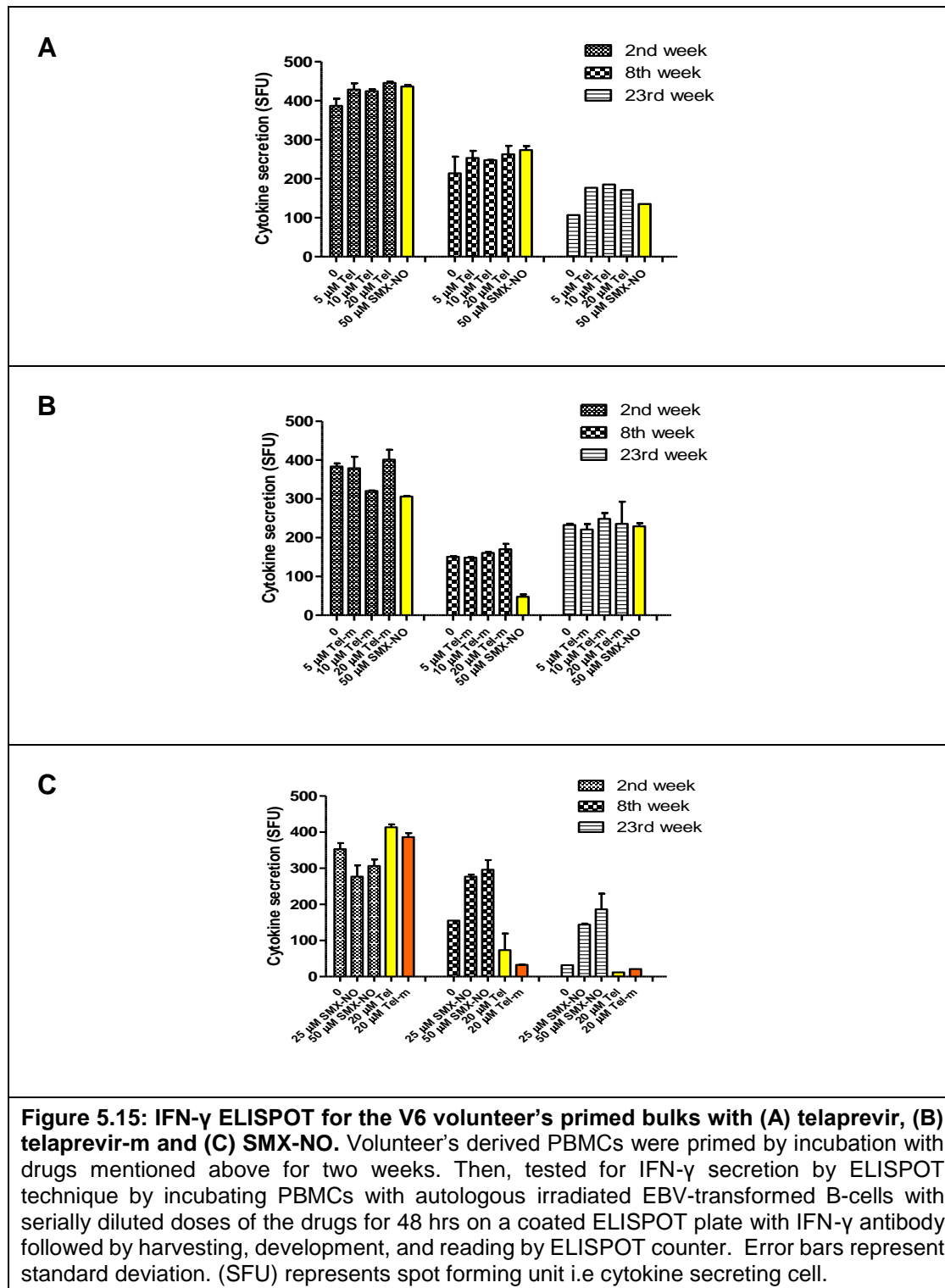
IFN- γ ELISPOT results for the three volunteers are shown in **Figure 5.13**, **Figure 5.14** and **Figure 5.15**. Similar to the proliferation results, telaprevir and telaprevir-m are negative with SIs lower than 1.5 with no clear dose dependent response.

For SMX-NO the picture shows stronger sensitivity in terms of SIs and dose response. In which there are higher SIs (≥ 1.5) for all volunteers.

In general, all the results were not significant as analyzed by Kruskal-Wallis test at $p < 0.05$ significance level.







5.3.4.3 Generation of telaprevir and telaprevir-m specific clones from telaprevir and telaprevir-m treated PBMCs

Clones were generated from telaprevir and telaprevir-m treated PBMCs from all 3 volunteers using serial dilution. Clones were then tested for telaprevir or telaprevir-m specificity using a ^3H -thymidine uptake proliferation assay.

Clones responsive to telaprevir and telaprevir-m were not generated with the exception of one patient. Twenty telaprevir-m reactive clones (out of 232 clones tested) were generated from volunteer V5 (**Figure 5.16**). $\text{SI} \geq 2$ were regarded as positive.

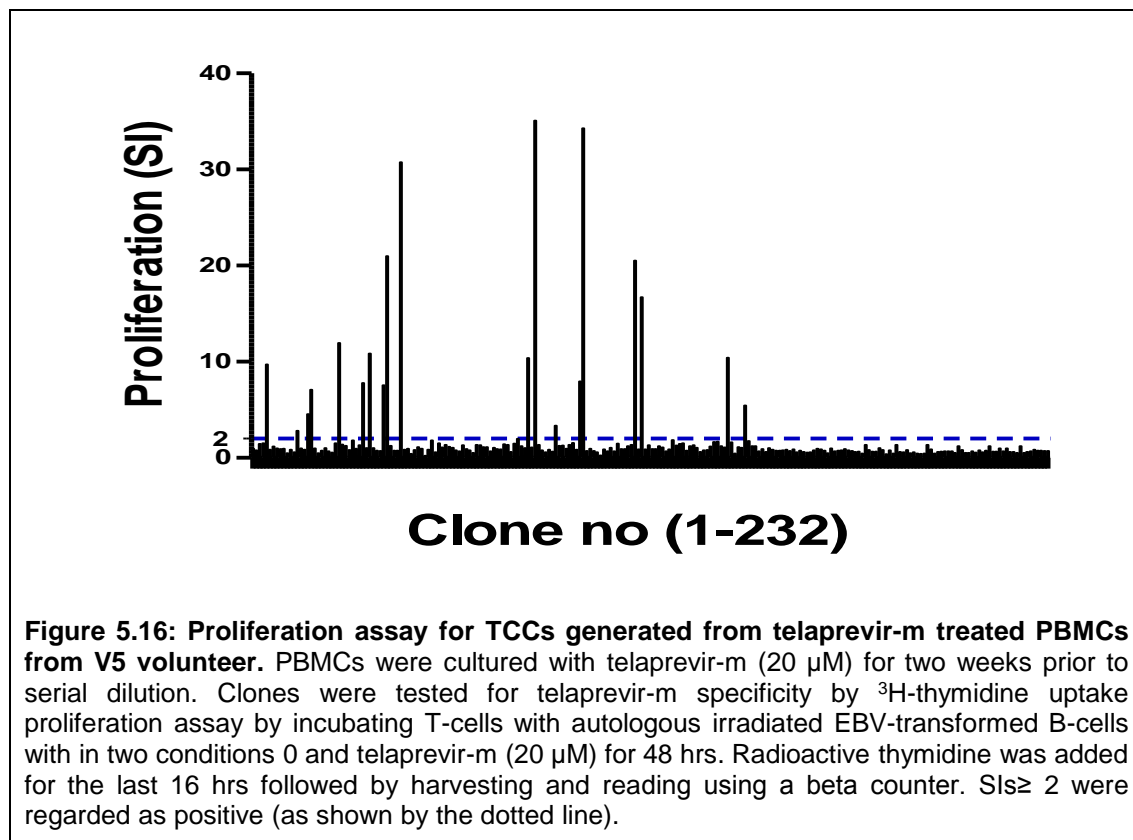


Figure 5.16: Proliferation assay for TCCs generated from telaprevir-m treated PBMCs from V5 volunteer. PBMCs were cultured with telaprevir-m (20 μM) for two weeks prior to serial dilution. Clones were tested for telaprevir-m specificity by ^3H -thymidine uptake proliferation assay by incubating T-cells with autologous irradiated EBV-transformed B-cells with in two conditions 0 and telaprevir-m (20 μM) for 48 hrs. Radioactive thymidine was added for the last 16 hrs followed by harvesting and reading using a beta counter. $\text{SIs} \geq 2$ were regarded as positive (as shown by the dotted line).

5.3.4.4 Measurement of the crossreactivity of telaprevir-m responsive clones using ^3H -thymidine uptake proliferation assay

The 20 telaprevir-m responsive clones were tested using ^3H -thymidine uptake proliferation assay with serially diluted doses of telaprevir, telaprevir-m and SMX-NO (50 μM) as an irrelevant antigen. Fifteen clones proliferated in a dose-dependent manner in the presence of telaprevir-m. 100% cross reactivity was observed between telaprevir and telaprevir-m. The strength of the response initiated with both compounds was identical. Crossreactivity between these drugs and SMX-NO was not observed (**Figure 5.17**). Statistical significance was observed when all concentrations of telaprevir and telaprevir-m were compared with the vehicle control (analyzed Kruskal-Wallis test at $p \leq 0.05$ significance level).

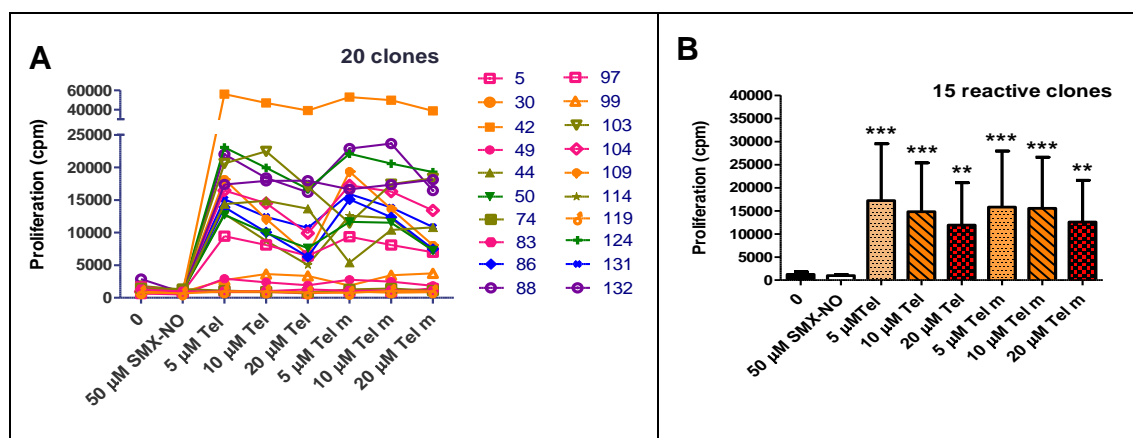
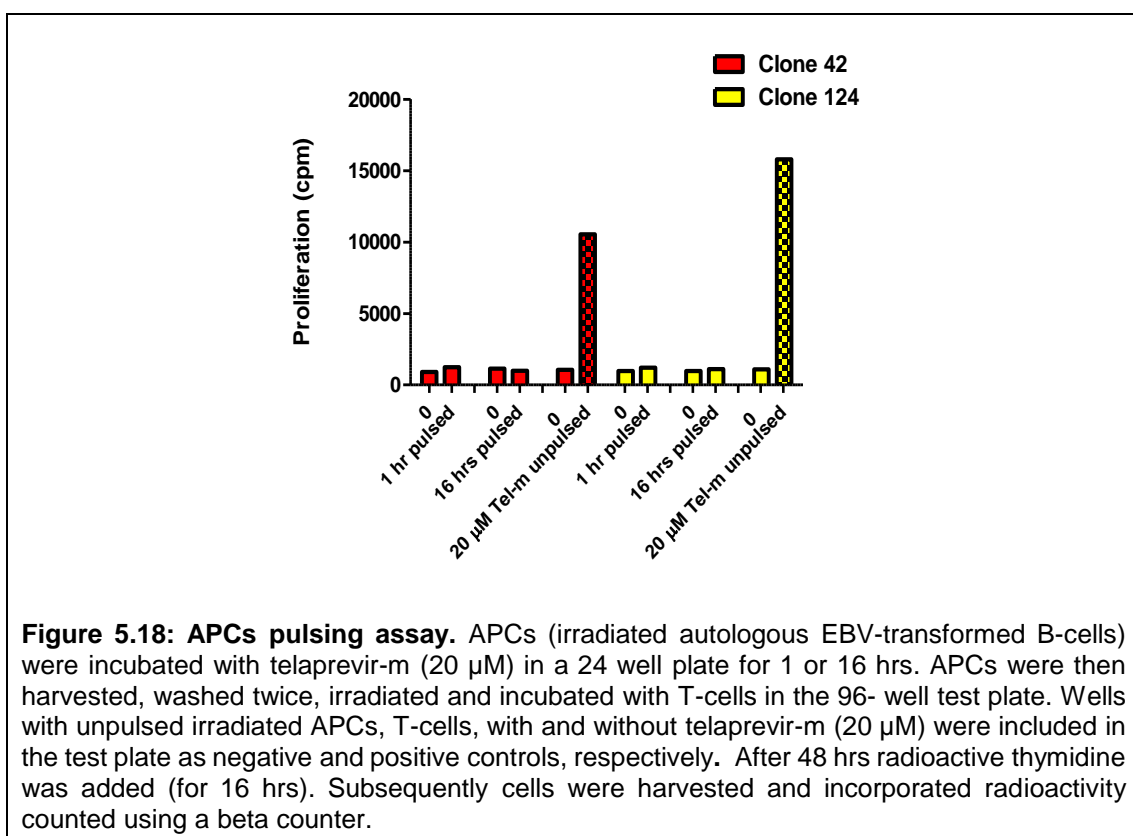


Figure 5.17: (A) ^3H -thymidine uptake proliferation on serially diluted doses of telaprevir and telaprevir-m showing 20 clones individually from V5 volunteer. **(B)** The mean proliferative response of 15 selected reactive clones. T-cells were incubated with autologous irradiated EBV-transformed B-cells with serially diluted doses of telaprevir and telaprevir-m for 48 hrs. Radioactive thymidine was added at last 16 hrs followed by harvesting and reading by beta counter. Proliferation was determined by radioactive thymidine incorporation. Statistical analysis using Kruskal-Wallis test reveal high statistical significance ** and *** signifying $p < 0.01$ and $p < 0.001$ respectively. Error bars represent standard deviation. (cpm) represents the count per minute (i.e. radiation unit).

5.3.4.5 APCs pulsing assay

Two clones were tested for their sensitivity to telaprevir-m in the presence of telaprevir-m pulsed autologous irradiated EBV-transformed B-cells (**Figure 5.18**).

Clones (42 and 124) were not stimulated to proliferate when exposed APCs pulsed with telaprevir-m for 1 or 16 hrs. Soluble telaprevir-m was used as a positive control. Both clones proliferated vigorously with soluble drugs (SI=10.02, SI=14.57 respectively).

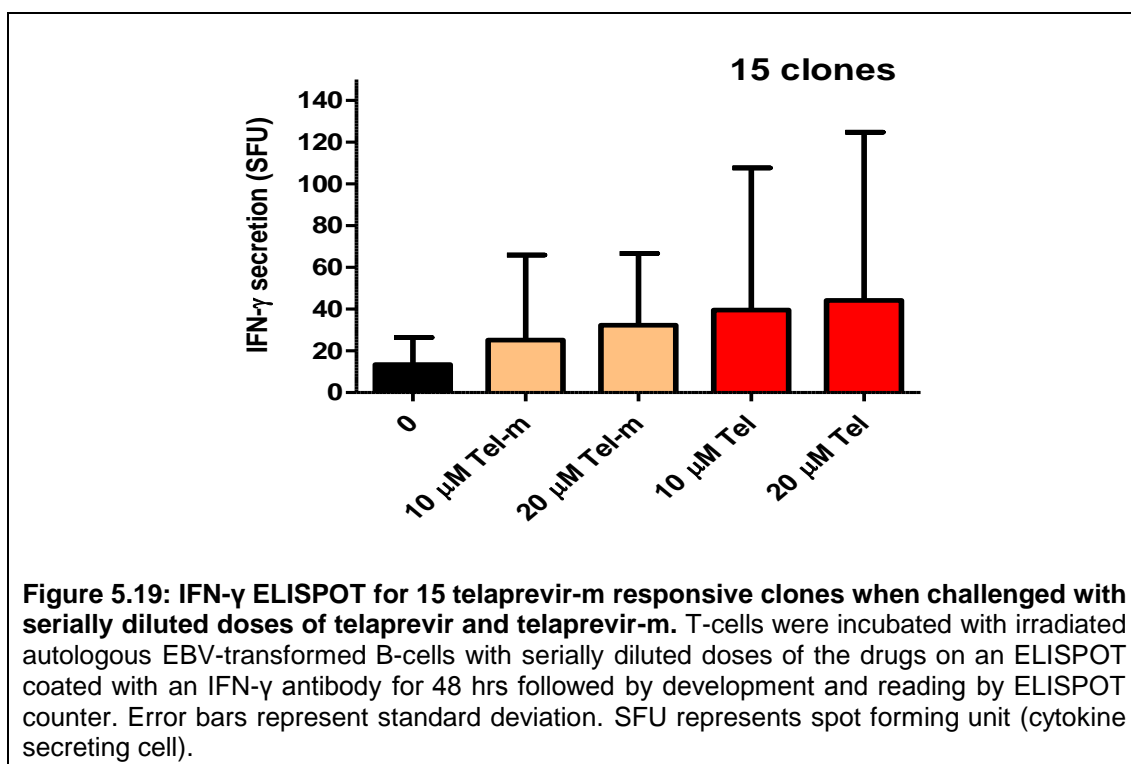


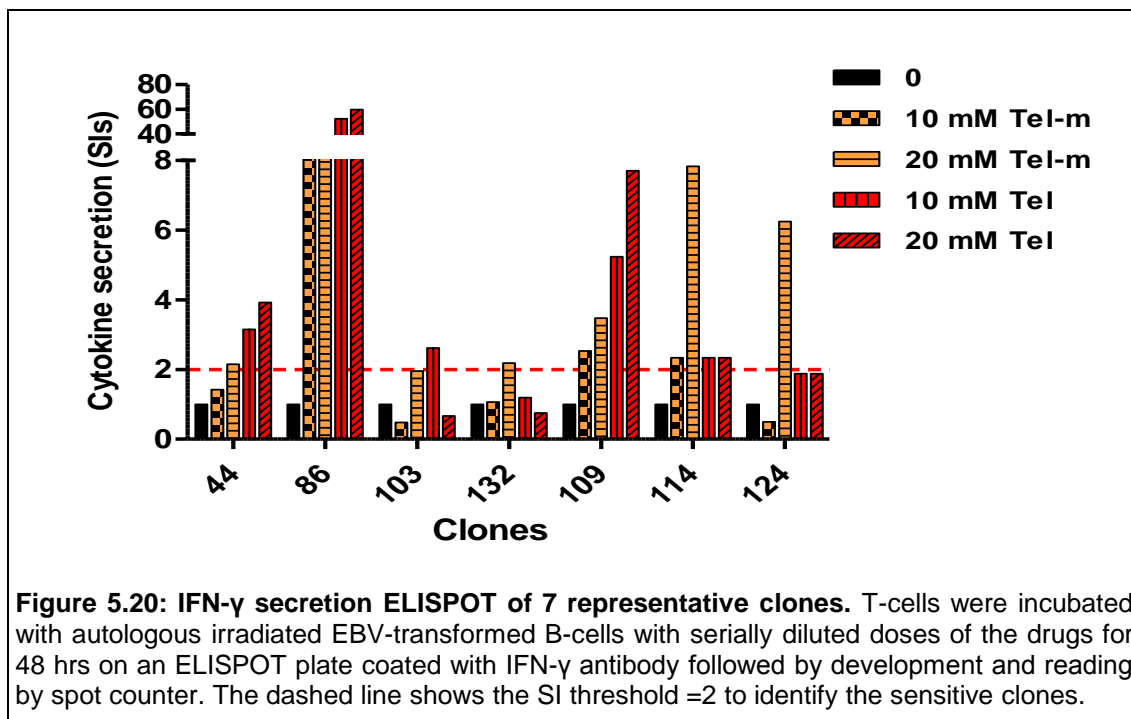
5.3.4.6 Qualitative and quantitative measurement of telaprevir-m clones in terms of sensitivity and specificity (cross reactivity) using IFN- γ ELISPOT technique

The 15 clones that proved to be sensitive in the proliferation assay were tested for IFN- γ secretion using the ELISPOT technique.

Many clones secreted IFN- γ in response to telaprevir and telaprevir-m in a dose-dependent manner (**Figure 5.19**). A high degree of cross reactivity was observed between telaprevir and telaprevir-m. However, due to the variation between clones, no statistical significance was observed when the combined data was analyzed using Kruskal-Wallis test.

Detailed analysis of 7 clones showed that 2 clones (132, 124) secreted IFN- γ in response to telaprevir-m alone, while the other 5 clones responded in the presence of both telaprevir and telaprevir-m to various extents (**Figure 5.20**).





5.3.4.7 Measurement of T-cell receptor expression using the Fluorescence-activated cell sorting (FACS)

- **CD4/CD8 phenotype, CD45RO expression (memory phenotype)**

Thirteen telaprevir sensitive clones were analyzed using flow cytometry for their CD4/CD8 phenotype and CD45RO expression (memory phenotype).

10 clones were shown to be CD4+ (76.9%) while 3 clones were CD8+ (23%) (**Figure 5.21**). All clones were CD45RO+, i.e., memory T-cells.

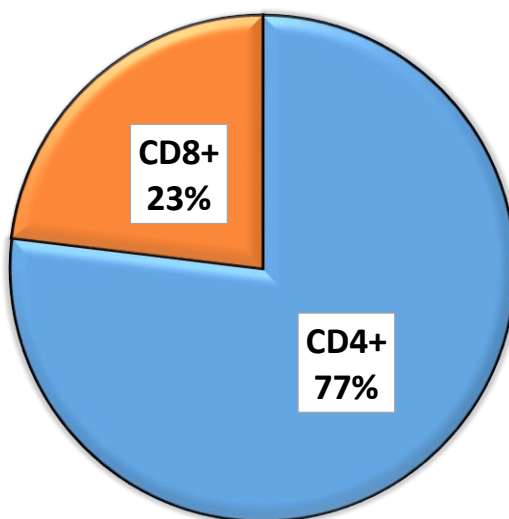


Figure 5.21: CD4/CD8 analysis by FACS for 13 telaprevir sensitive clones. (50 μ L) T-cells suspension was stained with (3 μ L) of CD3, CD4, CD8 and kept on ice (at 4C°) for (20 min) in FACS tubes. Then, FACS buffer was added followed by centrifugation. Cells were resuspended in FACS buffer (200 μ L). Then, analyzed using flow cytometry.

- **TCR-V β expression**

Thirteen telaprevir sensitive clones were analyzed for TCR-V β expression using flow cytometry. The analysis showed a restricted pattern of TCR-V β in which there was a high expression of TCR-V β 22 (n=6, 46%), TCR-V β 2 (n=5, 38%) and TCR-V β 5.1 (n=1, 8%). One clones did not express any TCR-V β in the antibody panel (**Figure 5.22**).

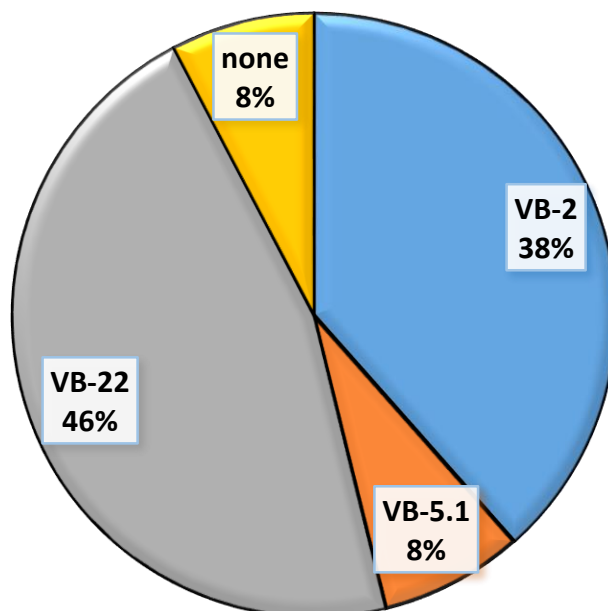
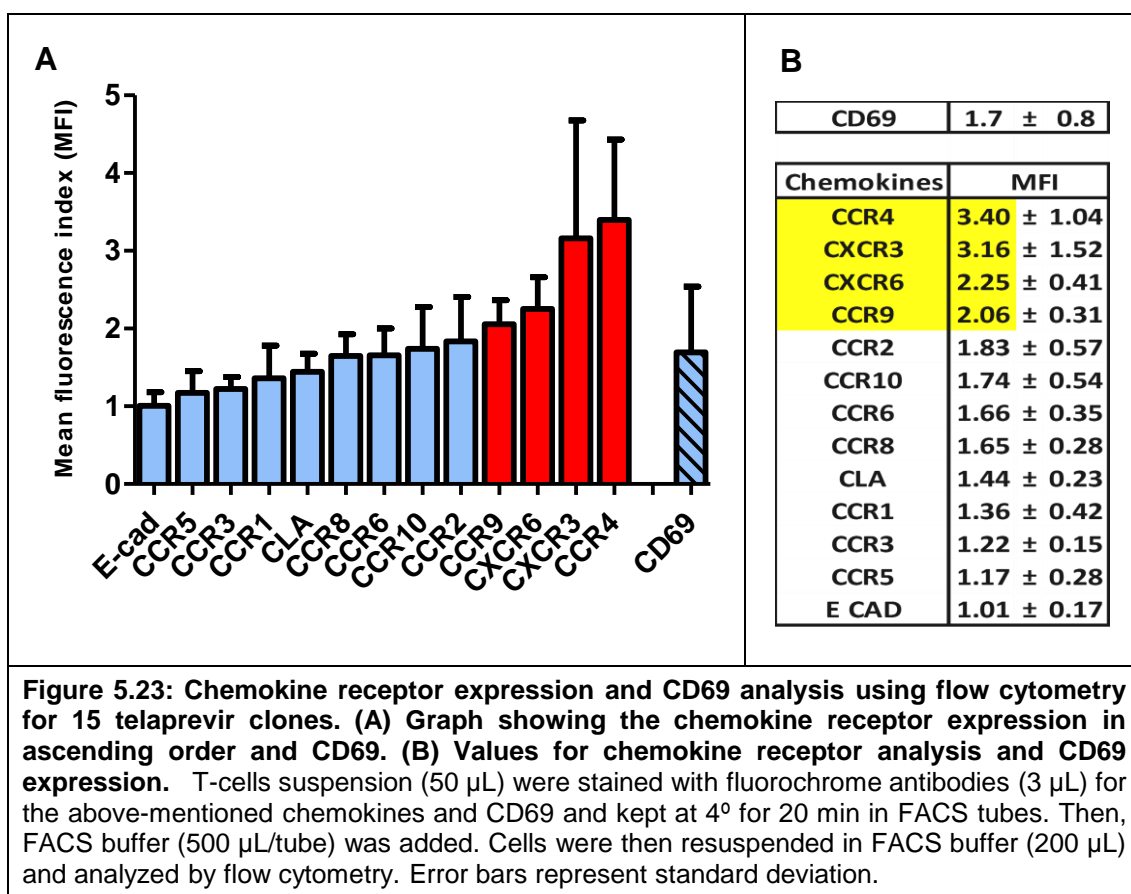


Figure 5.22: TCR-V β expression of 13 TCCs using FACS method. (50 μ L) T-cells suspension were stained with (5 μ L) fluorochrome antibodies for TCR-V β and kept at room temperature for (20 min) in FACS tubes. Then, (500 μ L/tube) FACS buffer was added followed by centrifugation. Then, cells were resuspended in (200 μ L) FACS buffer. Finally, the cells were analyzed using FACS machine.

5.3.4.8 Characterization of chemokine receptors and CD69 expression on telaprevir-m responsive T-cells clones:

Fifteen telaprevir-m responsive clones were tested for their expression of chemokine receptors and CD69 using flow cytometry.

High expression of the following chemokine receptors was observed (all data presented in terms of MFI): CCR4 3.40 ± 1.04 , CXCR3 3.16 ± 1.52 , CXCR6 2.25 ± 0.41 and CCR9 2.06 ± 0.31 . CD69 expression was 1.7 ± 0.8 (**Figure 5.23**).



5.4 Discussion

Telaprevir hypersensitivity was identified relatively soon after widespread administration of the drug. Reactions have a delayed onset and are observed at a therapeutic dose of the drug, which both indicate that the adaptive immune system may be involved in the disease pathogenesis. A high incidence of mild skin reactions is observed in patients exposed to traditional treatments for hepatitis C (i.e. pegylated interferon- α and ribavirin); however, the frequency of reactions is increased when telaprevir is administered. Furthermore, a small number of patients develop severe reactions such as Stevens Johnson syndrome and toxic epidermal necrolysis. Since pharmacogenetic studies failed to identify an association between expression of a specific HLA alleles and development of mild or severe reactions, the objective of this chapter was to investigate if it was possible to identify telaprevir-responsive T-cells in healthy drug-naïve donors. Since telaprevir readily converts to its isomer in patients, T-cell responses against telaprevir and this primary metabolite were studied.

The first problem that we faced was to identify the optimal dose for priming and testing T-cells with telaprevir and telaprevir-m. Thus, we carried out a toxicity assay which revealed that both drugs inhibited mitogen-driven T-cell proliferation at the same concentration (i.e. 50 μM). Thus, we chose two doses (20 μM , 5 μM) for priming T-cells against both telaprevir and telaprevir-m and to monitor the response using proliferation and cytokine release as markers of T-cell activation.

For the purpose of comparison with the clinical data, a study on Japanese HCV patients have shown that the mean C_{max} for telaprevir was (5.4 μM) at a steady state (Yamada et al., 2012). For the unbound fraction, the C_{max} was approximately (2 μM) (Kwong et al., 2011). Telaprevir-m maximal plasma concentrations at the steady state were found to be almost equivalent to those of telaprevir (Nakada et al., 2014). Thus, the lower concentration used in T-cell assays was similar to that circulating in patient plasma.

Two approaches have been described in the literature to detect drug-specific T-cells in healthy naïve donors and these are summarized below.

- *Drug-stimulation of pre-activated memory T-cells* (Engler et al., 2004): Peripheral blood lymphocytes undergo several rounds of antigen-driven stimulation using peripheral blood mononuclear cells as antigen presenting cells. The aim of this protocol is to expand the number of antigen-responsive T-cells prior to analysis of antigen-specificity.
- *Drug stimulation of naïve T-cells* (Faulkner et al., 2012): This assay relies on the isolation and culture of highly pure T-cell and antigen presenting cell populations. Immature monocyte-derived dendritic cells and naïve T-cells are used as antigen presenting cells and responder cells, respectively. After 8-day culture period, T-cells are re-exposed to the drug antigen and dendritic cells and antigen specificity is measured shortly after.

Both assays were used to detect telaprevir and telaprevir-m responsive T-cells.

The proliferation assay is widely used since it is highly applicable with many different drugs in different immune reactions that are mediated by drug specific T-cells. In addition, with high sensitivity (approximately 76%) and specificity (95%) it can be used to measure the priming of T-cells in both quantitative and qualitative ways (Pichler, 2014). On the other hand, IFN- γ ELISPOT is highly sensitive assay that can detect 10-1000 SFU per million PBMCs. Moreover, IFN- γ ELISPOT is especially important in measuring Tc1 (cytotoxic) and Th1 (helper) responses (Smith et al., 2001). Thus, two assays were used following T-cell priming to detect T-cell responses after the primed cells were restimulated with autologous antigen presenting cells (PBMCs) and the relevant drug antigen.

PBMCs from hypersensitive patients are expected to contain drug-specific T-cells as sensitization occurs at the time of drug exposure and just prior to the hypersensitivity reaction. In contrast, PBMCs from drug-naïve healthy volunteers

should not be activated with the drug and this is indeed the case. PBMCs from healthy volunteers are not activated to at least 10 drugs antigens commonly used in Liverpool. Moreover, factors like a specific HLA allele or TCR-V β profile might make the T-cells from hypersensitive patients more sensitive to the culprit drug.

We have noticed from the previous two chapters that patients derived PBMCs were more productive in terms of the number of clones generated as compared with naïve healthy volunteers.

Thus, blood samples were collected from three telaprevir-naïve healthy volunteers. PBMCs were separated and then naïve T-cells primed using the established DC-T-cell priming assay. Despite multiple attempts, telaprevir- and telaprevir-m-responsive T-cells were not detected in proliferation or ELISPOT assays. Furthermore, TCCs were generated from telaprevir-treated T-cells; however, none of the clones were stimulated to proliferate with telaprevir or telaprevir-m.

SMX-NO a well-established drug antigen was used as an irrelevant drug antigen with cells from each volunteer. In agreement with data presented in chapter 3, naïve T-cells were activated with SMX-NO. Restimulation of the primed T-cells resulted in a dose-dependent proliferative response and the release of IFN- γ . Moreover, it is obvious that there was no cross reactivity between SMX-NO and telaprevir or telaprevir-m.

The reason behind non-productivity of DC-T-cell priming could be attributed to the volunteer selection. Volunteers vary in their criteria regarding HLA, TCR-V β , age, sex and many other parameters that were discussed in the previous chapters. Thus, T-cell responses might have been detected if an increased number of volunteers were studied. However, it is also possible that telaprevir does not activate naïve T-cells. As what has been shown with abacavir recently, the drug might interact with HLA molecules expressed on antigen presenting cells to stimulate pre-existing memory T-cells. For this reason, we also utilized the second culture method outlined above, which uses PBMCs and not purified naïve T-cells.

PBMCs were cultured with telaprevir, telaprevir-M and SMX-NO for several weeks. At various time-points the T-cell lines were tested for their drug-specificity using proliferation and IFN- γ secretion as readouts over several variable dates for the purpose of optimising the assay with the final target to get the highest yield in terms of sensitivity and specificity of the bulks for telaprevir or telaprevir-m.

For the volunteers 4 and 5 there was no SMX-NO cultures at the start of the priming because there were not enough cells to prepare all the priming sets for these volunteers. However, following a second blood donation, SMX-NO-specific T-cell lines were established. Similar to the data discussed above, we were generally unsuccessful at generating telaprevir and telaprevir-m-responsive T-cell lines.

However, lines generated from V5 and V6 showed very weak T-cell responses to telaprevir. Thus, we attempted to generate telaprevir and telaprevir-m sensitive clones from for the T-cell lines of all volunteers V4, V5 and V6 using the serial dilution technique.

Twenty CD4+ and CD8+ clones out of 232 clones generated were stimulated to proliferate with telaprevir-m. Clones displayed 100% cross-reactivity with telaprevir and drug stimulation was also associated with the secretion of IFN- γ . Telaprevir-m-pulsed antigen presenting cells did not activate the clones; thus, the drug likely binds directly to MHC molecules expressed on the surface of antigen presenting cells to activate the cells via a p-i mechanism. Clones expressed different T-cell receptor V β , which indicates that they do not originate from a common precursor. Finally, the clones were not activated with the structurally irrelevant antigen SMX-NO.

A marked expression of the following chemokine receptors was found on the clones: CCR4, CXCR3, CXCR6 and CCR9. CCR4 and CXCR3 are skin homing receptors (Restifo et al., 2013). This would provide an explanation for the patho-mechanism underlying skin reactions in telaprevir hypersensitivity. Telaprevir specific T-cells expressing CCR4 and CXCR3 would migrate to skin to exert their

actions. CXCR3 is expressed by Th1 T-cells while CCR4 is expressed by Th2 T-cells (Kim et al., 2001). These findings suggest a mixed population of Th1 and Th2 T-cells populations.

CCR9 is well known marker as a gut homing receptor for T-cells (Agace, 2008). It has a role in the development of small and large intestinal inflammation. Moreover, CCR9 antagonists have been found to be effective in ulcerative colitis treatment (Bekker et al., 2015). Intestinal involvement in case of telaprevir hypersensitivity is very common [diarrhoea (25%), ano-rectal discomfort (11%) and Haemorrhoids (12%)]. The underlying mechanism of intestinal disruption is not known. However, topical steroids are indicated in the management of intestinal involvement (Pockros, 2012), which may give a clue that an immune pathogenesis linked with high levels of CCR9 expression and T-cells activation.

Notably, CXCR6 is also elevated on the drug specific T-cells. CXCR6 has a role in T-cell migration to the liver (Heesch et al., 2014). Another study has described a role for CXCR6 in chronic inflammatory conditions (Wilbanks et al., 2001).

The most notable observation in this study was that telaprevir has been shown for the first time to activate T-cells. Clones were generated from 1 out of 3 volunteers studied. It could be that this donor would be susceptible to telaprevir hypersensitivity if exposed to the drug. However, many more volunteers would need to be studied to directly relate the frequency of T-cell responses detected *in vitro* to the incidence of reactions observed in exposed patients. Our data also highlights the limitation of existing T-cell priming assays using cells from healthy volunteers. Both assays lacked the sensitivity to detect telaprevir-responsive T-cells. Finally, in on-going experiments researchers in Liverpool are utilizing other metabolites of telaprevir to attempt to define the major antigenic determinants that activate T-cells.

Chapter Six

Final Discussion

6 Final Discussion

The objective of this work was to investigate mechanisms of drug specific T-cell activation. The project focuses for the most part on the β -lactam antibiotic piperacillin for three reasons. First, clinical samples from a well-defined patient cohort were available; second, T-cells have been isolated from patients previously and shown to be drug-specific; finally, mass spectrometric methods are established to characterize drug-protein binding in in vitro cell culture. Thus, it is possible to relate the chemistry of drug antigen formation to the activation of T-cells from hypersensitive patients. Piperacillin-specific T-cells cloned from the hypersensitive patients were used to explore whether the parent drug and/or drug protein adducts stimulate proliferative responses and cytokine release via ligation of specific T-cell receptors. T-cell clones are useful to work with as all cells derive from the same precursor and hence express the same T-cell receptor. Clones can be expanded using mitogens from a single cell up to approximately 10×10^6 cells, which allows for detailed mechanistic and phenotypic characterization. Hence, it was also possible to characterize the clones in terms of T-cell receptor and chemokine receptor expression.

One of the major problems that we faced in the study was the diminished sensitivity of clones over time which is a well-recognized phenomenon that has been noted previously (Pauken and Wherry, 2015). In general, TCCs retain their specificity and are able to respond in proliferation and cytokine release assays for about 2 months. However, their activity shows downward trend and the duration of time when experiments can be conducted varies from clone to clone. T-cell clones are incubated in culture medium, which although supplemented with nutrients and kept in optimal temperature and CO₂ levels, is still an unnatural environment. Consequently, they become exhausted, which manifests in the form of poor proliferation and/or poor cytokine secretion. Therefore, restimulation of the clones was performed on periodic schedule. Nevertheless, T-cells expansion

does not mean necessarily that the newly generated cells have a higher or even similar reactivity to their ancestors in terms of sensitivity (although they do retain the same specificity). Previous research has shown that repeated stimulation leads to T-cells exhaustion and ultimately a non-responsive state (Pauken and Wherry, 2015).

Thus, all of the studies described in this thesis were conducted in a narrow time window when the clones were optimally responsive. When different numbers of clones were used in individual experiments this was almost always due to T-cell exhaustion.

6.1 Free piperacillin and conjugated piperacillin

Since Landsteiner first wrote about drug haptens in 1935, there have been great leaps forward in our understanding of drug hypersensitivity, resulting in two main theories to explain pathways of drug-specific T-cell activation: the hapten hypothesis and p-i mechanism.

We have tried to broaden the knowledge of drug hypersensitivity reactions especially regarding the role of drug-protein conjugates in the activation of T-cells. In this thesis, we have focused on β -lactam hypersensitivity since all β -lactam antibiotics form protein adducts through the irreversible modification of specific amino acid residues on proteins such as human serum albumin (HSA). The β -lactam ring of drugs such as piperacillin is attacked by lysine residues of proteins leading to ring opening and binding of penicilloyl groups. Previous studies have shown the pivotal role of drug-protein adducts in T-cells activation (Thierse et al., 2005;Ortmann et al., 1992;Weltzien et al., 1996).

The results described in chapters 3 and 4, show that piperacillin hypersensitive patients with CF display two distinct T-cell responses: one involves free piperacillin and the other piperacillin-HSA conjugates. Both sets of T-cell clones show comparable features in terms of cytokine secretion, chemokine expression and CD4/CD8 phenotype. However, there was a difference in TCR-V β

expression, which was diverse for free piperacillin-responsive clones, but more restricted for clones activated with piperacillin-HSA adducts.

Both sets of clones are activated by a pathway dependent on APCs protein processing (as shown by APCs fixation assays). Thus, the activation of both sets of clones seems to involve a hapten mechanism. Pulsing assays using free piperacillin have shown that clones are activated upon incubation with APCs that have been incubated with free piperacillin for long durations (24-48 hrs). This is the time required to generate high levels of protein adduct in cell culture (Whitaker et al., 2011). Similar lengths of incubation were required for T-cell clones to respond to piperacillin-HSA adducts. This likely relates to the time required for protein processing and the liberation of MHC binding peptides. The lowest level of piperacillin-HSA modification required to trigger T-cells was found to be 2.8% which correlates well with piperacillin-HSA adducts levels detected in serum of patients exposed to a 14-day course of treatment (2.7-4.7% [mean 3.86%]). Collectively, these data show that T-cell stimulatory levels of drug protein adduct are formed in plasma of almost all patients exposed to piperacillin. Since the majority of patients exposed to the drug do not develop hypersensitivity, additional factors must be required to translate the antigenic signal into an immune response and tissue injury. The nature of these signals is not known, but may relate to disease status at the time of the hypersensitivity reaction. For example, infection is known to result in activation of important signalling pathways the innate immune system. Furthermore, innate immune signalling is critical for the activation of naïve T-cells to drug and protein antigens.

Clones responsive to free piperacillin did not cross react with piperacillin-HSA conjugates while clones responsive to piperacillin-HSA conjugates clones showed some reactivity to free piperacillin. Incubation of free piperacillin leads to modification of lysine residues of HSA but at a low percentage for pip-K541 peptide and for a shorter duration of time, which is evidently not sufficient to produce a T-cell proliferative response or cytokine secretion. On the other hand,

the percentage of modified lysine residues in (250:1) piperacillin-HSA conjugates is approximately (25%) and very strong proliferative responses of clones were observed.

Piperacillin-HSA conjugate sensitive clones are not cross reactive with HSA conjugates of other β -lactam antibiotics although they share the same β -lactam core ring. Thus, the β -lactam core ring per se does not contribute to the fine specificity of the clones. It is likely that the major antigenic determinant is the piperacillin-modified peptide formed after APC processing of piperacillin-HSA conjugate.

Given that TCCs responsive to free piperacillin are activated via a processing dependent pathway, it was unexpected when cross-reactivity with piperacillin-HSA conjugates was not observed. There are two hypotheses to explain this:

- ***1st hypothesis: Each set of clones has a unique piperacillin antigenic determinant***

This is supported by the discrepancy in TCR-V β expression between the two sets of TCCs. Piperacillin is known to modify extracellular proteins in in vitro cultures, but ignores intracellular proteins (Monshi, 2013;El-Ghaiesh et al., 2012;Meng et al., 2011). This selectivity is restricted to β -lactam antibiotics. Other drugs, like SMX, are known to modify multiple intra and extracellular proteins (Naisbitt et al., 2002;Sanderson et al., 2007;Megherbi et al., 2009;Callan et al., 2009).

By implementing novel spectrometric methods, we were able to identify albumin as the main protein modified by piperacillin binding and identify the profile of piperacillin binding at specific lysine residues in terms of dose and incubation time. Since the majority of serum bound penicilloyl groups are bound to human serum albumin, we have considered it as a model protein for investigating piperacillin-HSA conjugates formation. Our results confirm that these conjugates are formed in piperacillin treated patients and are capable of inducing the host T-cell immune response. Although piperacillin-HSA conjugates are found in 100% of piperacillin

treated patients, they do not result in a pathogenic immune response unless other factors (patient related) are present.

The in vitro culture medium is supplemented with human AB serum. Thus, it contains an array of other serum proteins, albeit and significantly lower levels when compared with albumin. It is possible that clones circulating in the periphery of hypersensitive patients are activated by different drug-modified peptides and the peptides likely originate from different proteins.

- ***2nd hypothesis: Both sets of TCCs share the same piperacillin antigenic determinant***

It is possible that the antigenic determinant for clones (whether free piperacillin or piperacillin-HSA conjugate responsive) is the same (i.e., a piperacillin-modified peptide). This hypothesis is supported by the finding of high cross reactivity of the piperacillin-HSA clones with free piperacillin. Thus, in ongoing experiments, peptide elution studies are being conducted to explore the nature of the culprit antigenic determinants that bind to MHC molecules in different hypersensitive patients.

Role of piperacillin metabolism

Piperacillin is metabolized by the liver to form desethyl piperacillin (approximately 20% of total piperacillin) which is known to form adducts with serum proteins, which may play a role in mediating piperacillin hypersensitivity reactions (Whitaker et al., 2011). Thus, there is a need to study the role of desethyl piperacillin and its protein adducts.

As we have shown in chapter 4, piperacillin forms two kinds of adduct with HSA; cyclized and hydrolysed variants. Desethyl piperacillin HSA adducts have also been detected in plasma of patients exposed to the drug; however, the significance of these metabolite-modified proteins in piperacillin hypersensitivity needs to be investigated.

Role of other nucleophiles

Although studies on β -lactam antibiotics have characterized a binding interaction with lysine residues that results in the formation of penicilloyl adducts, we cannot exclude the possibility that other nucleophiles such as histidine, serine and cysteine are also modified. Previous studies have found no evidence of other modified amino acid residues (Meng et al., 2011). However, as mass spectrometric methods develop, minor adducts that might have a massive influence on the immune response might be detected. Furthermore, it is possible that these adducts are too labile and therefore undetectable using existing analytical methods (TSUJI et al., 1975).

Piperacillin conjugation with other proteins

As mentioned above, it is likely that piperacillin binds to other serum proteins and the subsequent liberation of piperacillin-modified peptides may activate certain T-cells from hypersensitive patients. Piperacillin may bind to immunologically active molecules such as cytokines as in the case of benzyl penicillin which makes adducts with IFN- γ , IL-1B, IL-2, IL-5, IL-13, TNF- α (as shown by western blotting assays). Interestingly, many of these cytokines are secreted by piperacillin-specific T-cells (Whitaker et al., 2011). Hence, it is possible that piperacillin-HSA adducts activate the primary T-cell response in patients that leads to the secretion of pro-inflammatory cytokines. Piperacillin cytokine binding might generate novel antigenic determinants, which would spread the repertoire of drug-responsive T-cells in hypersensitive patients and potentially extend to duration or severity of the iatrogenic disease.

Site of T-cell priming

- **Blood or skin?**

In all the experiments described in this thesis, I worked on blood-derived T-cells. However, there is now extensive evidence that the T cells implicated in drug hypersensitivity reactions are actually derived from tissues e.g. skin as in SJS

(Schrijvers et al., 2015). Thus, we can address a question: Would our findings showing activation of clones from blood of hypersensitive patients with free piperacillin and piperacillin-conjugated HSA be different if we have used T-cells derived from skin?

In research conducted by Gaide et al, it has been shown that skin derived T-cells are similar to blood derived T-cells in terms of peptide antigen specificity in a particular individual. Moreover, skin, lymph nodes and blood derived T-cells were shown to descend from a common naïve ancestor and they express similar TCR-V β expression (Gaide et al., 2015). Although these data are somewhat preliminary and require replication in different model systems, it seems to suggest that blood- and skin-derived clones will display similar antigen specificities.

Characterisation of skin-derived free piperacillin-responsive T-cells by Sullivan et al (unpublished data at the University of Liverpool) have shown a comparable T-cell cytokine secretion profile. However, they have found that skin T-cells showed a greater cytolytic activity; specifically, high levels of perforin, granzyme-B and Fas ligand secretion were detected with the skin-derived clones. Moreover, blood- and skin-derived clones were found to secrete high levels of the tissue-specific cytokine IL-22 when stimulated with the drug.

- **Role of B-cells**

For a broader, more detailed understanding of the immune mechanisms implicated in piperacillin hypersensitivity, there is a need to address the pivotal role of B-cells.

B-cells synthesise specific antibodies to culprit antigens. These antibodies could be IgE and thus induce Type I hypersensitivity reactions or IgG and therefore could activate complement system or induce cells lysis through the ADCC mechanism (Pollara et al., 2011).

In research conducted in Liverpool in parallel to my investigations, B-cells from piperacillin hypersensitive patients showed signs of activation with high

expression of CD19 and CD27 (Amali, 2015). Moreover, there was a high level of piperacillin-specific IgG in vivo as well as in vitro. In contrast, IgG secretion from B-cells derived from tolerant volunteers and healthy controls was at undetectable levels after piperacillin stimulation. These IgG molecules are highly specific to piperacillin i.e. they did not bind to benzyl penicillin, penicillin V, amoxicillin and aztreonam protein adducts which provides further evidence of the difference in antigenic determinants between piperacillin and other antibiotics. Importantly, the role of B-cells in the skin injury associated with piperacillin-mediated drug hypersensitivity reactions is not known. B-cells might impact on the severity or nature of the skin reaction; however, it is theoretically possible that the drug-specific antibodies might down-regulate the cytotoxic T-cell response through restricting APC access to drug protein adducts.

Collectively, the findings of my study provide a step forward in the understanding of piperacillin hypersensitivity reactions and also drug hypersensitivity reactions in general. The hypotheses, methods and analytic interpretation can be used as a model for future studies on other drugs. Moreover, this data may impact on the design of safer and more effective drugs. In addition, an understanding of the characteristics of drug antigens will contribute positively to the development of new diagnostic and predictive screening methods.

6.2 Telaprevir and its metabolite

Since the FDA approved use of telaprevir for the treatment of genotype 1 chronic hepatitis C in 2011, many reports were made about the side effects of telaprevir. These side effects plus the discovery of newer, safer and cheaper agents for treating hepatitis C have altogether contributed to the decision of the parent company Vertex Pharmaceuticals to discontinue telaprevir production.

However, it is important to investigate why the drug was associated with such a high incidence of frequently severe hypersensitivity reactions. Furthermore, telaprevir might represent a model for other compounds in the same chemical

class i.e. protease inhibitors for hepatitis C (e.g., boceprevir and semiprevir). This research may also improve our understanding of hypersensitive reactions in general.

Since telaprevir-m (VRT-127394) is a major metabolite, we chose to include it in our study alongside the parent drug to explore T-cell responses to both compounds.

Telaprevir has previously been shown to promote the secretion of cytokines when antigen presenting cells were exposed to the drug at relatively high concentrations for a prolonged period of time. However, the ability of telaprevir to activate T-cells has not been investigated. Difficulties in recruiting patients with hypersensitivity to telaprevir led us to design in vitro environments with cells from healthy donors. Two methods were used to identify telaprevir-responsive T-cell clones.

DC-T-cell priming vs PBMCs priming

The mechanism of T-cell priming differs greatly between the two methods that were employed. This variation is due the different type of the APC used in each assay.

In the DC-T-cell priming assay, antigen presentation is dependent on DCs (DCs to naïve T-cells ratio=1:31), whereas antigen presentation in PBMC priming is mediated by multiple cells that make up PBMCs. This includes DCs (0.04×10^6 cells/mL), B-cells (0.3×10^6 cells/mL) and macrophages and monocytes (0.55×10^6 cells/mL) (Stemcell, 2015). Thus, the ratio of DCs: naïve T-cells in PBMCs in vitro culture would be 1:13.5 which is greatly different from the DC-T-cell priming ratio.

There is a difference in the MHC II density on the different APCs, there are approximately (45500 molecules) on each DC while approximately (25000 molecules) are expressed on B-cell (Shibuya et al., 2015) and macrophages express even fewer molecules (Kumar, 2016). However, there are no significant differences in the number of MHC class I molecules between the three main APCs (DCs, B-cells and the macrophages) (Kumar, 2016). As MHC molecules play a

pivotal role in antigen presentation, it is clear why DCs are so important for activating T-cells. Furthermore, dendritic cells are the only cells known to have the ability to efficiently prime naïve T-cells (Mellman and Steinman, 2001).

In DCs priming the naïve T-cells are the only source of drug-specific T-cells (since we have implemented bead separation to remove other cell types). While in PBMC priming, there are two possible sources of drug-specific T-cells. They are either:

- Naïve T-cells (like DCs priming).
- Memory T-cells which were primed by previous drug exposure (which does not apply here since the study was performed on naïve healthy volunteers PBMCs samples). However, there is still a possibility that drug-specific T-cells cross reactive with a peptide antigen.

The incubation duration is limited to one week in DC-T-cells priming while it is two weeks in PBMCs priming. Furthermore, in PBMCs priming, the cells can be exposed to the drug several times in the presence of irradiated autologous PBMCs (as APCs).

In case of naïve T-cells, drug-mediated T-cell responses develop in the first 4-7 days (memory T-cells require 1-3 days) (Mak et al., 2013). of the remainder of the incubation period to allow the primed T-cells to expand. Thus, PBMCs priming technique has the privilege of allowing more time for T-cell expansion in addition to the boosting effect from the multiple rounds of restimulation.

In general, the priming assays (whether DC-T-cell or PBMCs priming) presented in chapter 5 were unsuccessful. No tangible difference between the two methods were detected; telaprevir-specific proliferative responses and IFN- γ secretion were not observed. Attempts to generate clones showed that using PBMCs priming was more efficient. A relatively large number of telaprevir-m specific clones were isolated from volunteer 5 using this method.

The detection of drug antigen specific T-cells in healthy volunteers was intriguing. As discussed above, it is difficult to determine whether drug-responsive T-cells derive from the memory or naïve compartment using the PBMC priming assay; however, the negative data with DC-T-cell priming suggests that telaprevir might activate pre-existing memory T-cells in healthy volunteers. In ongoing experiments, the protocol is being repeated using PBMCs depleted of naïve or memory T-cells to address this question. Similar conclusions were made about the abacavir in HLA-B*57:01+ naïve donors. Abacavir seemed to preferentially activate memory T-cells (Lucas et al., 2015).

Therefore, there is a need to conduct further research to explore the mechanism of telaprevir-specific T-cell activation and to define the number of individuals who have circulating T-cells that might be activated by the drug.

The data presented in this thesis have made a significant contribution to our understanding of immunological adverse drug reactions. Specifically, I have helped to delineate the pathomechanism of piperacillin hypersensitivity reactions in patients with CF. Moreover; my studies establish the framework for future studies characterizing antigenic determinants of culprit drugs. Regarding telaprevir, I believe that this study has shown for the first time that the drug activates T-cell proliferative responses and cytokine release. There is a need for further work on patients derived T-cells to complete the missing pieces in our understanding of telaprevir-mediated hypersensitivity reactions.

Chapter Seven

Appendix

7 Appendix

Table 7.1: Biological Reagents and Chemicals		
	Biological Reagent	Manufacturer
1	Amoxicillin	Sigma Aldrich
2	Anti HLA –ABC (purified NA /LE mouse)	BD pharmingen
3	Anti HLA –DR, DP, DQ (purified NA /LE mouse)	BD pharmingen
4	Antihuman CCR10 conjugated mouse IgG	R & D systems
5	Antihuman CCR2 conjugated mouse IgG	R & D systems
6	Antihuman CCR3 conjugated mouse IgG	R & D systems
7	Antihuman CCR4 conjugated mouse IgG	R & D systems
8	Antihuman CCR5 conjugated mouse IgG	R & D systems
9	Antihuman CCR6 conjugated mouse IgG	R & D systems
10	Antihuman CCR8 conjugated mouse IgG	R & D systems
11	Antihuman CCR9 conjugated mouse IgG	R & D systems
12	Antihuman CD69 conjugated mouse IgG	BD Biosciences
13	Antihuman CXCR10 conjugated mouse IgG	BD Biosciences
14	Antihuman CXCR3 conjugated mouse IgG	BD Biosciences
15	Antihuman CXCR6 conjugated mouse IgG	BD Biosciences
16	Antihuman E-cad conjugated mouse IgG	BD Biosciences
17	APC Mouse Anti-Human CD3	BD Biosciences
18	BCIP/NBT-plus	Mabtech AB
19	BD CompBeads	BD
20	Bovine serum albumin BSA	Sigma-Aldrich
21	Bradford reagent	BIO-RAD
22	Carboxyfluorescein succinimidyl ester (CFSE)	eBioscience Ltd.
23	CD14 Microbeads Human conjugated to monoclonal CD14 antibodies (isotype: mouse IgG2a)	Miltenyi Biotec
24	CD25 MicroBeads II human: MicroBeads conjugated to monoclonal anti-human CD25 antibodies (isotype: mouse IgG1).	Miltenyi Biotec
25	CD4 microbeads	Beckman Coulter
26	CD4 multisort kit	Beckman Coulter
27	CD45RO MicroBeads, human: MicroBeads conjugated to monoclonal mouse anti-human CD45RO antibodies (isotype: mouse IgG2a).	Miltenyi Biotec
28	CD8 microbeads	Beckman Coulter
29	CD8 multisort kit	Beckman Coulter
30	Cy5.5 mouse antihuman CD45ro	BD pharmingen
31	Cyclosporin-A (98.5%)	Fluka Analytical
32	dichloromethane	Sigma Aldrich

33	dimethylformamide	Sigma Aldrich
34	DMSO	Sigma-Aldrich
35	Ethylene diamine tetra acetic acid disodium salt solution [EDTA] 0.5M	Sigma-Aldrich
36	Fetal bovine serum	Gibco, Invitrogen
37	FITC Mouse Anti-Human CD4	BD Biosciences
38	FITC mouse antihuman CD45ra	BD pharmingen
39	Flucloxacillin	CP pharmaceuticals ltd
40	Fmoc-L-amino acids	Sigma Aldrich
41	Glutaraldehyde solution	Sigma Aldrich
42	Glycine	Sigma Aldrich
43	Hank's buffered salt solution [HBSS]	Sigma-Aldrich
44	Heat inactivated pooled human AB serum	Innovative research
45	HEPES 1M	Sigma-Aldrich
46	Human GM-CSF	Peprtech
47	Human AB serum	Innovative Research
48	Human FasI ELISPOT kit	Abcam
49	Human Granzyme B ELISPOT kit	Mabtech AB
50	Human IFN- γ ELISPOT kit	Mabtech AB
51	Human IL-13 ELISPOT kit	Mabtech AB
52	Human IL-5 ELISPOT kit	Mabtech AB
53	Human Perforin ELISPOT kit	Mabtech AB
54	Human serum albumin crystallized and lyophilized	Sigma-Aldrich
55	Interleukin (IL)-2	PeprTech EC Ltd
56	Interleukin (IL)-4	PeprTech EC Ltd
57	Isotype MHC I control (purified NA /LE mouse IgG)	BD pharmingen
58	Isotype MHC II control (purified NA /LE mouse IgG)	BD pharmingen
59	L-Glutamine	Sigma-Aldrich
60	Lyrnphoprep	Axis Shield
61	Monkey marmoset peripheral blood lymphocyte (B95-8 cell line)	Abd Serotec
62	Mouse antihuman CCR4 PE	BD pharmingen
63	Mouse antihuman CCR8 FITC	BD pharmingen
64	Mouse antihuman CCR8 PE	BD pharmingen
65	N, N'-Dicyclohexylcarbodiimide	Sigma Aldrich
66	N-methylmorpholine	Sigma Aldrich
67	PE Mouse Anti-Human CD8	BD Biosciences
68	Penicillin/streptomycin	Sigma-Aldrich
69	Penicillin-G sodium salt	Sigma Aldrich
70	Phosphate buffered saline (powder)	Sigma Aldrich

71	Phytohemagglutinin PHA-P, cell culture tested, lyophilized powder	Sigma-Aldrich
72	Piperacillin sodium salt	Sigma- Aldrich
73	Piperidine	Sigma Aldrich
74	RPMI 1640	Sigma-Aldrich
75	Secondary goat anti-mouse HRP conjugated antibody	Dako,
76	Skimmed Milk Powder	Sigma-Aldrich
77	SMX-NO	Dalton Pharma Services
78	Sodium azide	Sigma Aldrich
79	Streptavidine-ALP	Mabtech AB
80	Tetanus toxoid	Statens seruminstitut (Copenhagen, Denmark).
81	Transferrin	Sigma-Aldrich
82	Trifluoroacetic acid	Sigma Aldrich
83	Tritiated [3H]-methyl thymidine	Moravek
84	Trypan blue (0.2 % w/v)	
85	Trypsin	Promega
86	Tube A: TCR V β Kit (V β 5.3 PE, V β 7 PE+FITC, V β 3 FITC)	Beckman Coulter
87	Tube B: V β 9 PE, V β 17 PE+FITC, V β 16 FITC	Beckman Coulter
88	Tube C: V β 18 PE, V β 5.1 PE+FITC, V β 20 FITC	Beckman Coulter
89	Tube D: V β 13.1 PE, V β 13.6, PE+FITC, V β 8 FITC	Beckman Coulter
90	Tube E: V β 5.2 PE, V β 2 PE+FITC, V β 12 FITC	Beckman Coulter
91	Tube F: V β 23 PE, V β 1 PE+FITC, V β 21.3 FITC	Beckman Coulter
92	Tube G: V β 11 PE, V β 22 PE+FITC, V β 14 FITC	Beckman Coulter
93	Tube H: V β 13.2 PE, V β 4 PE+FITC, V β 7.2 FITC	Beckman Coulter
94	Tween®20	Sigma Aldrich
95	Western Lightning Chemiluminescence reagent	Perkin Elmer

Table 7.2: Labware utilized.		
	Lab ware	Manufacturer
1	AID ELISPOT reader	AID Diagnostika GmbH
2	C18 PepMap column	Dionex
3	Centrifugal concentrator	SpeedVac, Eppendorf
4	centrifugal filter	Amicon Ultra-15, Millipore
5	Culture tubes with caps (FACS Tubes) /polystyrene	Simport
6	Drying Oven	Sciquip
7	ECL sensitive film	Kodak
8	ELISPOT plates	Millipore
9	ELISPOT Reader	AID GmbH

10	FACS Canto II flow cytometer	BD Biosciences
11	Filters	Sartorius
12	Jupiter C18 column (10µm C18, 250 mm × 10mm)	Phenomenex
13	Leica DME microscope	Leica Microsystems
14	Lithium Heparinized Vacuette Tubes	Greiner Bio-One Ltd
15	LS Columns	Miltenyi Biotec
16	LS Columns	Miltenyi Biotec
17	Microbeta Trilux (beta Counter)	PerkinElmer
18	Microsealer WALLAC 1495-021	TWALLAC
19	MS Columns	Miltenyi Biotec
20	Neubauer haemocytometer	Sigma-Aldrich
21	Nitrocellulose membrane	GE Healthcare Life Sciences
22	Nitrocellulose membrane	Amersham Biosciences
23	PicoTip	New Objective
24	POROS anti-HSA affinity cartridge	Applied Biosystems, Foster City, USA
25	Printed Filtermat A	PerkinElmer
26	Quadrupole-linear ion trap mass spectrometer	Sciex
27	Sample Bag for Microbeta TM	PerkinElmer
28	T-175 flasks	Thermo Scientific
29	Tomtec Harvestor 96	Receptor Technologies
30	Transwell plates	Corning Incorporated
31	Vision Workstation	Applied Biosystems
32	Vivaspin Ultrafiltration tubes	Vivaproducts
33	Wax scintillant meltilex A	PerkinElmer
34	ZipTips	Millipore

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